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THE UNIVERSITY OF ALBERTA

PYRIMIDINE NUCLEOSIDE TRANSPORT IN ISOLATED RAT HEPATOCYTES

by

EMMANUEL M.K. AWUMEY

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

SPRING, 1989

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PYRIMIDINE NUCLEOSIDE TRANSPORT IN ISOLATED RAT HEPATOCYTES submitted by EMMANUEL M.K. AWUMEY in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

A R P. Paterson
.....
Supervisor

A Clarke
.....

Mallen
.....

David L. Tyrrell
.....

Date 29 March 1989

DEDICATION

To the memory of my father

ABSTRACT

Nucleosides and nucleoside analogues enter cells via nucleoside-specific transport systems of which several types are known. Nitrobenzylthioinosine (NBMPR), a potent inhibitor of equilibrative nucleoside transport (NT) systems, has been a powerful tool for exploration of these systems. The high-affinity binding of NBMPR to transporter-associated elements in the plasma membrane has been correlated with NT inhibition in a number of animal cells. NBMPR-insensitive NT systems have also been recognized in neoplastic cells and epithelia and some of these transporters have been identified as energy-dependent, concentrative systems. The present study has employed NBMPR in the characterization of systems in freshly-isolated rat hepatocytes that mediate the inward transport of uridine and thymidine.

In earlier studies from this laboratory, the presence of NT systems of low sensitivity to NBMPR ($IC_{50} > 1\ \mu M$), was recognized in several lines of cultured rat hepatoma cells. The present study demonstrates the presence in fresh hepatocytes from rats of (i) high-affinity NBMPR binding sites (K_D, 0.53 ± 0.08; B_{max}, 4.27 ± 0.42 x 10^5 sites/cell), and (ii) uridine and thymidine transport systems of high and low sensitivity to NBMPR, findings that suggest interaction between the NBMPR binding site complex and nucleoside transporters.

When hepatocytes were incubated at 22 °C with uridine or thymidine in medium containing Na^+ salts, cellular uptake of either nucleoside was concentrative. Cellular concentrations (nucleoside equivalents) of these nucleosides reached levels about twice those of the medium. Uridine inhibited the uptake of thymidine in these cells suggesting that the same mechanism may be involved in the permeation of these nucleosides into hepatocytes. Incubation of hepatocytes at 22 °C with 1 mM unlabelled thymidine enhanced the uptake of labelled thymidine. Both nucleosides were poorly metabolized in the hepatocytes and at least 80% of the cellular content of these agents was in the form of free nucleosides after 10 min of incubation at 22 °C.

Potassium ions were a poor substitute for Na^+ in supporting uridine and thymidine fluxes, however, replacement of Na^+ with N-methyl-D-glucammonium ion resulted in a six-fold

enhancement of thymidine uptake during 5 min of incubation, suggesting that the latter ion may take the place of Na^+ as a co-substrate for the thymidine transporter in these cells. The kinetics of uridine and thymidine influx indicated that more the one process contributed to the observed fluxes.

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ABBREVIATIONS

B_{max}	maximum number of ligand binding sites per cell as determined from equilibrium binding data by mass law analysis
Dilazep	3,4,5-trimethoxybenzoic acid diester with tetrahydro-1H-1,4-diazepine-1,4(5H)-dipropanol
Dipyridamole	2,2',2'',2'''-(4,8-dipiperidinopyrimido[5,4d] pyridine-2,6-diyldinitrilo)tetraethanol -2,6-diyldinitrilo)tetraethanol
dThd	thymidine
EDTA	ethylenediamine tetraacetic acid
HBS	HEPES-buffered saline
HEPES	N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid
Hexobendine	3,4,5-trimethoxybenzoic acid 1,2-ethanediylbis[(methylimino)3,1-propanediyl] ester
IC_{50}	concentration at which 50% inhibition occurs
K_D	dissociation constant
K_m	substrate concentration for half-maximal unidirectional flux (analogous to the Michaelis-Menten constant)
NEM	N-ethylmaleimide
NBMPR	6-[(p-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine (nitrobenzylmercaptopurine riboside)
NMG^+	N-methyl-D-glucammonium ion
NT	nucleoside transport
PBS	phosphate-buffered saline
pCMBS	p-chloromercuribenzene sulphonate
PEG	polyethylene glycol
PEI-cellulose	polyethyleneimino-cellulose
S-MEM	minimum essential medium with spinner salts
Thy	thymine

Ura	uracil
Urd	uridine
UV	ultraviolet
V_{max}	maximum velocity
VP-16	etoposide (an epipodophyllotoxin)

I. GENERAL INTRODUCTION

A. Nucleoside transport in mammalian cells: an overview

Fluxes of physiological nucleosides and of many nucleoside analogues[1] across the plasma membranes of mammalian cells are mediated by nucleoside-specific transport elements that are intrinsic components of the membrane (Paterson and Cass, 1986). At least three different types of carrier-mediated processes are now recognized as responsible for the movement of nucleosides across cell membranes. The most widely studied of these nucleoside transport (NT) systems is a bidirectional, non-concentrative system of broad specificity, sensitive to inhibition by specific NT inhibitors (Plagemann and Wohlhueter, 1980; Paterson *et al.*, 1981a, 1981b; Young and Jarvis, 1983). The best-studied NT system of this type is that of the human erythrocyte; the transport process mediated by this system is a classical, reversible "facilitated diffusion" process in which permeants move in one direction or the other across the plasma membrane down concentration gradients.

A number of cultured tumour cell lines express a second type of non-concentrative NT activity distinguished chiefly by its relative insensitivity to several inhibitors (Belt, 1983; Belt and Noel, 1985; Plagemann and Wohlhueter, 1985; Kaplinsky *et al.*, 1986). A third type of NT system, in which the transport process is concentrative and dependent on the trans-membrane Na^+ gradient has been recognized in a number of different systems (Ungemach and Hegner, 1978; Spector and Huntoon, 1984; Spector, 1985; Le Hir and Dubach, 1984 and 1985; Schwenk *et al.*, 1984; Darnowski and Handschumacher, 1986; Darnowski *et al.*, 1987; Belt and Vijayalakshmi, 1988; Vijayalakshmi and Belt, 1988). The Na^+-nucleoside co-transport system is of low sensitivity to nitrobenzylthioinosine (NBMPR) and dipyridamole, potent inhibitors of reversible NT systems of the erythrocytic type.

---

[1] The terms, "nucleoside analogue" and "nucleoside drug" refer to pentofuranosyl derivatives of (i) purines and pyrimidines bearing unphysiological groups, (ii)heterocyclic molecules that are analogues of the purine and pyrimidine ring systems, and (iii) various heterocyclic structures unrelated to the nucleobases.

Nucleoside fluxes mediated by the transport mechanisms described above show concentration dependence, saturability with respect to permeant concentration and are subject to competitive inhibition by other nucleosides, characteristics that identify them as carrier-mediated processes. The recognition that some cell types express both equilibrative and concentrative NT systems has added to the general appreciation of the complexity of cellular NT systems (Paterson *et al.*, 1987). The equilibrative NT systems in human erythrocytes and certain cultured cells show broad substrate specificity, accepting, as substrates, ribosides and deoxyribosides of the physiological purines and pyrimidines as well as many unphysiological nucleosides. The influx of adenosine and deoxyadenosine into these cells have low K_m values relative to other physiological nucleosides, a finding which suggests that the adenine nucleosides are "preferred" substrates for these NT systems (Paterson and Cass, 1986).

Pentofuranosides of several S^6-substituted 6-thiopurines and N^6-substituted adenine derivatives (Cass *et al.*, 1981), and the structurally unrelated compounds, dilazep (Paterson *et al.*, 1984) and dipyridamole (Lum *et al.*, 1979; Paterson *et al.*, 1980; Jarvis, 1986a) are potent inhibitors of equilibrative NT processes and have served as useful probes of these NT mechanisms. The best-studied of the pentofuranoside inhibitors is nitrobenzylmercaptopurine riboside (NBMPR) which binds with high affinity (K_D, 0.1-1.0 nM) to plasma membrane sites associated with nucleoside transporters in many cell types. NBMPR binding to sites on human erythrocytes and cultured S49 mouse lymphoma cells, both of which possesss NT systems of high NBMPR sensitivity ($IC_{50} < 10$ nM) correlates with inhibition of transport (Cass *et al.*, 1974 and 1981).

NT mechanisms in cultured neoplastic cells have been classified on the basis of their interactions with NBMPR. The association of NBMPR binding sites with NT elements of the plasma membrane is apparent in a large body of evidence and is well illustrated by the demonstration that an apparently single mutational event in S49 cells yielded AE_1 cells, a clonal line selected for adenosine resistance. The AE_1 mutation resulted in (i) general impairment of NT, (ii) elimination of site-specific binding of NBMPR (Cass *et al.*, 1981; Cohen *et al.*, 1979), and (iii) resistance to growth inhibition by cytotoxic nucleosides present

in culture medium (Cass *et al.*, 1981). These observations and the finding that certain lines of cultured cells are protected against otherwise cytotoxic concentrations of nucleoside analogues by the presence of transport inhibitors in the growth medium, show that simple diffusion plays a minor role in the permeation of these compounds in various cell lines (Paterson *et al.*, 1979a,b). However, the uptake of certain physiological nucleosides and nucleoside analogues occur by non-mediated processes. The lipophilicity of the nucleoside and the composition of the cell membrane influence these passive processes. In this laboratory, it has been shown that the entry of β-L-adenosine, the enantiomer of the naturally-occuring β-D-adenosine, in a human ovarian carcinoma cell line (HOC-7) proceeds by a transporter-independent permeation mechanism (Adjei and Paterson, 1988). NBMPR had no effect on the permeation process. The transport of 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxythymidine in human erythrocytes has also been shown to occur by non-mediated processes (Zimmerman *et al.*, 1987; Domin *et al.*, 1988).

B. Facilitated diffusion NT processes

The most widely studied NT system is that of human erythrocytes, a facilitated diffusion carrier with broad permeant specificity. Nucleoside fluxes in this system depend upon substrate concentration gradients across the plasma membrane. The initial rate of the transporter-mediated process is a function of substrate concentration and is saturable. This transport system is also subject to competition by structurally related compounds so that the transport of a particular nucleoside may be inhibited by other nucleosides as well as by various specific inhibitors of NT (Paterson *et al.*, 1981a, 1981b). These features demonstrate the existence of transporter-mediated permeation processes as opposed to permeation by simple diffusion (Stein, 1986). The facilitated diffusion systems that mediate nucleoside permeation in many cell types show broad specificity, transporting physiological nucleosides as well as nucleoside analogues (Paterson and Cass, 1986).

C. Sodium-dependent, concentrative NT processes

Although equilibrative NT systems have been extensively studied, the participation of the Na^+-dependent, concentrative systems in nucleoside fluxes across the plasma membrane in particular cell types have also been recognized. The transport of thymidine into isolated rat hepatocytes has been shown to occur by (i) a high-affinity, active transport process with narrow substrate specificity, and also by (ii) a low-affinity process having a broad substrate specificity (Ungemach and Hegner, 1978). A sodium-dependent, active transport system for thymidine and deoxyuridine influx has been described in isolated rabbit choroid plexus and the participation of a facilitated diffusion system in the efflux of these nucleosides has been observed (Spector and Huntoon, 1984; Spector, 1985). Schwenk *et al.*, (1984) have described a sodium-dependent, concentrative uridine transport in guinea-pig enterocytes. The transport of purine and pyrimidine nucleosides into renal brush border vesicles prepared from rat kidney was concentrative and dependent on extravesicular sodium ion (Le Hir and Dubach, 1984 and 1985). In mouse splenocytes, uridine was transported inwardly against the permeant concentration gradient and free uridine pools were built up (Darnowski and Handschumacher, 1986; Darnowski *et al.*, 1987). In mouse intestinal epithelial cells, formycin B, an analogue of inosine, and thymidine were transported by two different sodium-dependent systems (Vjayalakshmi and Belt, 1988).

In this laboratory, it has been shown that in monolayers of IEC-6 cells (derived from rat intestinal epithelium), the transport of formycin B was Na^+-dependent and concentrative (Jakobs and Paterson, 1986). Those cells possess membrane sites that bound NBMPR with high affinity (K_D, 1.4 ± 0.3 nM; B_{max} 1.5 x 10^6 sites/cell), indicating the presence of an NBMPR-sensitive NT system in addition to the energy-dependent system. In the absence of NBMPR, the time course of formycin B (a poorly metabolized nucleoside permeant) uptake was curved, but in the presence of NBMPR the rate was nearly constant over the period of observation. This result suggested that back-flux of formycin B occured as cellular levels increased and that an NBMPR-sensitive transporter mediated the outflow of formycin B. In cultured L1210 leukemia cells, a concentrative, sodium-dependent transport system

contributed to the entry of formycin B (Dagnino *et al.*, 1987). The energy-dependent transport system for nucleosides exhibits high affinity for substrates and may be widely distributed. It may, therefore, serve in the vectorial movement of nucleosides across epithelial cell layers and in the maintenance of nucleoside levels in plasma.

D. Transport and metabolism of nucleosides: tandem processes

NT systems accept, as substrates, both physiological nucleosides and analogs thereof. "Transport" refers to the transporter-mediated passage of permeant molecules across the plasma membrane. It is the initiating step in the multi-step process of nucleoside uptake. In this context, the term "uptake" refers to the cellular accumulation of a nucleoside permeant and its metabolic products, some of which may eventually be incorporated into nucleic acids (Paterson and Cass, 1986). Time courses of the cellular accumulation of nucleosides are commonly employed in the measurement of influx of nucleosides into cells. Upon entry into cells, nucleosides are metabolized by phosphorylation, deamination or phosphorylytic cleavage. Phosphorylation removes internalized physiological nucleosides from transport equilibria, effectively "trapping" them intracellularly because of the low permeability of the plasma membrane toward nucleotides (Plunkett and Cohen, 1977). Transport, the first step in the cellular uptake of nucleosides, proceeds independently of the metabolic events to which influent molecules are subject. The reversible transport step is rapid in many cell types and, under some conditions, may be faster than the subsequent metabolic transformations of the permeating molecules. Thus, in permeant-metabolizing cells, nucleoside uptake is the resultant of two independent processes operating in tandem; a reversible, facilitated diffusion step followed by an essentially irreversible, kinase-mediated phosphorylation step. Equations that describe the two processes have been developed and appear to adequately account for the biphasic nature of the time courses of nucleoside uptake by cultured cells (Heichal *et al.*, 1978; Koren *et al.*, 1979; Wohlhueter and Plagemann, 1980).

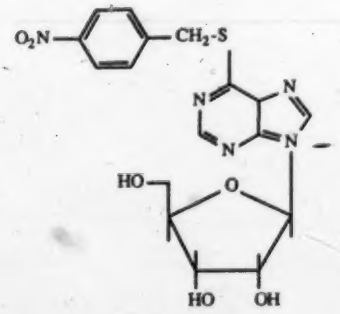
Regardless of the complex determinants of the time course of permeant uptake by cells, the initial rate of the uptake process is intrinsically the rate of inward transport of the

permeant (Paterson *et al.*, 1981a). In cells that metabolize nucleoside permeants, time courses of cellular accumulation of permeant are the resultant of influx and efflux processes, and metabolic removal of intracellular permeant from transport equilibrium. Since nucleoside fluxes are rapid in animal cells, the development of rapid sampling techniques was required in order to obtain definitive time courses of nucleoside uptake (Oliver and Paterson, 1971; Wohlhueter *et al.*, 1978a; Paterson *et al.*, 1984).

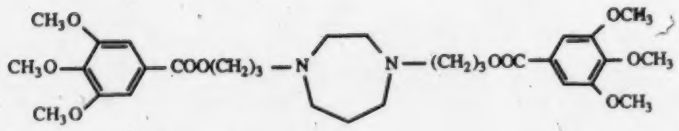
E. Inhibitors of nucleoside transport

NT inhibitors have been exceedingly valuable probes in the exploration of NT systems in various cell types (Plagemann and Wohlhueter, 1980; Young and Jarvis, 1983; Paterson *et al.*, 1983). Members of two families, the pentofuranosides of S^6-substituted 6-thiopurines and N^6-substituted adenine derivatives are potent inhibitors of nucleoside transport (Paterson *et al.*, 1981a, 1983). The most extensively studied compound in this group is NBMPR (Fig. 1) which binds tightly (K_D, 0.1-1.0 nM) to specific cellular sites, evidently on NT elements of the plasma membrane. NBMPR occupancy of such sites blocks nucleoside transporter function in human erythrocytes and S49 mouse lymphoma cells (Cass *et al.*, 1974; Lauzon and Paterson, 1977; Dahlig-Harley *et al.*, 1981). Using NBMPR binding to identify the nucleoside carrier, a polypeptide from human erythrocyte membranes presumed to be the carrier was partially purified and characterized as a band 4.5 polypeptide with a relative molecular weight of 45,000 - 65,000 (Jarvis and Young, 1981; Young *et al.*, 1983 and 1984). Cultured cells are protected from the toxic effects of various nucleoside analogues when NBMPR is present in the medium (Paterson *et al.*, 1979b; Cass *et al.*, 1981).

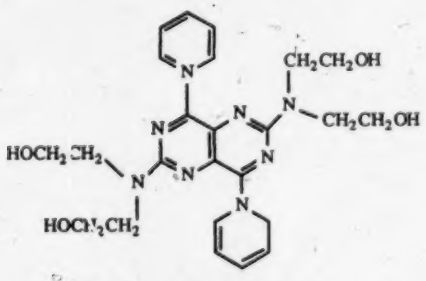
Although there is good evidence to support the idea that NBMPR binding sites are located on NT polypeptides in erythrocytes, relationships between NT elements and binding sites in nucleated cells are not clearly defined. Genetic experiments with S49 cells, in which mutations were introduced at the NBMPR site without impairment of transporter function, indicated that the binding and transport sites are genetically distinct (Aronow and Ullman, 1985; Aronow *et al.*, 1985; Cohen *et al.*, 1985).



NITROBENZYLTHIOINOSINE



DILAZEP



DIPYRIDAMOLE

Fig. 1: Structural formulae of three potent inhibitors of NT.

The possibility that the two sites might be separate is also supported by the demonstration of NT activity in cells that lack NBMPR binding sites (Belt and Noel, 1985; White *et al.*, 1985). In this laboratory it has been shown that, although NBMPR binding sites are present on cells of three cultured lines of rat hepatomas, their nucleoside transport systems are of low sensitivity ($IC_{50} > 1$ μM) to NBMPR (Ng, 1986; Paterson *et al.*, 1987).

A number of vasodilators, including dipyridamole (Lum *et al.*, 1979; Jarvis, 1986a), dilazep (Pohl and Brock, 1974), hexobendine (Kolassa *et al.*, 1978b) and lidoflazine (Van Belle, 1970), which bear no obvious structural relationships to the nucleosides, are also known to inhibit NT and the site-specific binding of NBMPR. A third group of compounds which inhibit nucleoside transport are the thiol reagents, NEM and pCMBS, and the natural products, cytochalasin B and VP-16 (Plagemann and Wohlhueter, 1980; Belt and Noel, 1985). The mechanism of NT inhibition by NEM and pCMBS appears to involve their interaction with essential thiol groups in the transporter protein.

F. The sensitivity of NT systems to NBMPR

At present, NT systems can be broadly classified into two groups in terms of sensitivity to NBMPR. In human erythrocytes and cultured S49 mouse lymphoma cells, NT systems are of high sensitivity to NBMPR[2]. NBMPR site occupancy and inhibition of NT in human erythrocytes are directly proportional (Cass *et al.*, 1974), and transport capacity in erythrocytes from several species is proportional to the abundance of binding sites on the cells (Jarvis *et al.*, 1982). In recent findings, it has been shown that NBMPR binding sites are present on nucleated cells of several cell lines that possess NT systems of low NBMPR sensitivity. Genetic evidence indicates that NT and NBMPR inhibition of transport in S49 cells are distinct functions. That conclusion derives from experiments showing that mutant clones of these cells are sensitive to nucleoside-mediated cytotoxicity in the presence of

[2] In this presentation, nucleoside transport systems of "high" NBMPR sensitivity will refer to those in which IC_{50} values were less than or equal to 10 nM, and "low" NBMPR sensitivity to systems in which IC_{50} values for NBMPR inhibition were equal to or greater than 1 μM.

NBMPR, while the parent line was protected by NBMPR from the toxic nucleosides (Aronow *et al.*, 1985). In wild-type Novikoff hepatoma cells, which are largely deficient in NBMPR binding sites, NT processes are of low sensitivity to NBMPR (Plagemann and Wohlhueter, 1984). Cells of the Novikoff UA line possess NBMPR binding sites, but NT is virtually unaffected by NBMPR binding to these sites (Gati *et al.*, 1986). These characteristics have been confirmed in Novikoff UASJ-2.9 cells, a clone in which cells lack NBMPR binding sites, but have NT capability. Another cloned line of Novikoff cells, the UASJ-5, has been shown to lack NBMPR binding sites, but have NT capability (Gati and Paterson, 1989). The equilibrative NT system of cultured Walker 256 carcinosarcoma cells is of low NBMPR sensitivity and these cells are devoid of NBMPR binding sites (Belt and Noel, 1985; Paterson *et al.*, 1987). In addition to these examples, cells of several rat hepatoma lines (Morris hepatoma 3924A, the Reuber hepatoma H-35 and Novikoff UASJ-2) express NT activity with low NBMPR sensitivity, yet have high affinity inhibitor binding sites (Paterson *et al.*, 1987).

In cloned lines of L1210 mouse leukemia cells (Belt, 1983) and in HeLa S3 cells (Dahlig-Harley, 1981), relationships between NBMPR concentration and nucleoside transport inhibition are biphasic. Belt (1983) suggested that this complex NBMPR effect could be attributed to the joint presence in these cells of NT systems of both high and low NBMPR sensitivity. This pattern has also been shown in several uncloned lines of cultured cells (Plagemann and Wohlhueter, 1984). Sites that bind NBMPR with high affinity are present on these cells and their occupancy by the inhibitor appears to relate to inhibition of NT systems of high NBMPR sensitivity.

G. NBMPR as a probe for nucleoside transporter function

NBMPR has been a valuable tool in exploring the biology and function of NT systems in animal cells of various types. Studies with human erythrocytes, S49 mouse lymphoma cells and AE_1 cells have led to conclusions that (i) high affinity sites which bind NBMPR represented NT elements, and (ii) binding site occupancy by the ligand inactivated NT activity (Cass *et al.*, 1974; 1981). Thus, capacities for transport by equilibrative, NBMPR-sensitive

NT systems could be derived from binding, assuming a fixed ligand:binding site stoichiometry, perhaps a 1:1 ratio between bound ligand molecules and sites. Comparison of NT processes in AE_1^- cells and cells of the parent S49 line has provided a clear demonstration that expression of NBMPR binding activity is under control of a single genetic element. Another finding that supports such an idea is the demonstration that sheep erythrocytes of a particular allelotype (Nu^+, Nu^+) possess high affinity NBMPR binding sites and transport nucleosides, but cells from sheep of another allelotype (Nu^+, Nu^-) are devoid of binding sites and are also incapable of nucleoside transport (Jarvis and Young, 1978; 1980; 1982). The abundance of NBMPR binding sites in erythrocytes from different species was shown to correlate with the uridine transport capacity of these cells, and differences in transport capacity from one cell type to another were due primarily to differences in the cellular number of functional nucleoside transporters (Jarvis *et al.*, 1982). These observations favour the view that high affinity binding sites for NBMPR represent functional NT elements of high NBMPR sensitivity. High affinity NBMPR binding has been reported in crude membrane preparations from guinea pig heart (Hammond and Clanachan, 1983), liver (Wu and Young, 1984) and in isolated hepatocytes from rat (Plagemann and Wohlhueter, 1985). Erythrocytes from rabbit, mouse, rat and guinea-pig have also been shown to possess high affinity NBMPR binding sites, which are also present on nucleated cells of various types and from many tissues. Thus, it appears that NBMPR-sensitive NT systems are widely distributed. However, it is now clear that the relationship between NBMPR binding site occupancy and NT inhibition in many cell types is more complex than was previously apparent. This complexity deepens as more NT mechanisms are discovered and more complex cell systems are studied.

H. Hepatocytes

The functional liver lobule is composed of parenchymal cells (hepatocytes), endothelial cells, and Kupffer cells (Guyton, 1981). Hepatocytes represent about 65% of the cells in the adult rat liver and, being larger than the other liver cells, they comprise 80-90% of

the total liver mass (Drochmans *et al.*, 1975). In the normal, human adult liver there are about 2.5×10^{11} hepatocytes (Arias *et al.*, 1982). Although their function, structure and shape depend on position in the liver plate, particularly in the various zones of the acinus, each hepatocyte probably has the potential for all hepatocyte functions. Hepatocytes located around the portal venules, for example, are morphologically different from those located around the hepatic venules, and cells linking these areas are intermediate between the two, although no anatomical boundaries exist between the three zones (Gumucio and Miller, 1981; Jungermann and Katz, 1982).

Collagenase-isolated hepatocytes have been found to express the functions observed *in vivo* (Gumucio *et al.*, 1986), and they provide a simple model for studying processes in the liver because they retain many of the properties demonstrable *in vivo* (Sirica and Pitot, 1980). Their advantages stem from their relatively long viability in culture, their homogeneity and the easy manipulation of environment. Having intact membranes and functioning drug-metabolizing systems, collagenase-isolated hepatocytes provide an approach to investigations of membrane transport and metabolic processes in liver. Suspension cultures and monolayer cultures of rat hepatocytes have been employed in transport and metabolism studies.

I. Preparation of parenchymal liver cells

Proteolytic and matrix-dissolving enzymes have been utilized in the preparation of intact cells from a variety of tissues. Collagenase has been found to be suitable for converting liver parenchymal tissue to suspensions of mostly intact cells. The perfusion of rat liver by a two-step procedure involving (i) Ca^{2+} removal and probably, removal of Ca^{2+}-dependent adhesion factor as well, and (ii) collagenase treatment, yields viable hepatocytes (Seglen, 1973 and 1976; Schwarze *et al.*, 1986). The collagenases are capable of solubilizing fibrous collagen by peptide cleavage under physiological conditions. This enzyme family includes specific bacterial collagenases that break the substrate into relatively small peptide fragments, and mammalian collagenases that split collagen molecules into only two fragments. Crude

collagenase from *Clostridium histolyticum* is a mixture of hydrolytic enzymes and other proteins including collagenases, an aminopeptidase, a trypsin-like endopeptidase and a neutral protease. This preparation has been used to isolate hepatocytes from rats (Seglen, 1976).

Collagen, which comprises 30% of the body protein in mammals, is the main constituent of skin, tendon and cartilage, and the main organic component of teeth and bone. In tropocollagen, the rod-like structural subunit of collagen, three parallel polypeptide chains of the same size are wound about each other in a left-handed poly-L-proline helix. It is also found in the extracellular matrix in association with glycoproteins and glycosaminoglycans. Several different types of collagen are known and each serve specific physiological functions in particular tissues. Type I collagen, which is abundant in liver, is found in portal triads, large vessels and around venules. Type II, which is the cartilage type of collagen, is absent from liver, and is found in lungs and eyes. Human liver contains approximately 33% of Type III collagen, which is present in portal triads and in terminal venule zones associated with Type I collagen. Type III collagen forms the fine, external framework of the liver and is present in interlobular regions. Type III collagen is also present in blood vessels, embryonic skin, lung and muscle (Rojkind, 1982).

In order to isolate cells for primary culture or other purposes, the matrix holding the hepatocytes together in the liver must be effectively broken down with minimal alteration to cell surfaces or intracellular structures (Jeejeebhoy and Phillips,1976). The introduction of collagenase as a liver cell-dispersing enzyme greatly facilitated the preparation of intact hepatocytes (Howard *et al.*, 1967). High yield preparation of intact liver cells became possible with the use of liver perfusion methods to make the tissue uniformly accessible to the action of collagenase (Berry and Friend, 1969).

The technical difficulties in the preparation of intact cells by perfusion with collagenase has led to a variety of methods for liver cell preparation. Numerous modifications at various steps in the preparation procedure have been introduced, however, "collagenase perfusion" is essentially one method. The initial cell suspension obtained after collagenase perfusion contains, in addition to intact parenchymal cells, variable numbers of

non-parenchymal cells and damaged cells, some cell clumps, pieces of connective and vascular tissue, and subcellular debris. Procedures used in the isolation of parenchymal cells include; (i) incubation of the initial cell suspension at 37 °C to complete structural damage, to allow parenchymal cells with irregular outline to assume a more rounded shape, and to reduce aggregation, (ii) filteration through gauze or nylon with special mesh sizes, (iii) differential centrifugation to remove smaller non-parenchymal cells and the much lighter, damaged parenchymal cells, (iv) separation of cell types using centrifugation techniques based on cell size (zonal or velocity sedimentation) or cell density (isopycnic gradient centrifugation). The latter technique is useful in the separation of intact and damaged hepatocytes (Seglen, 1976; Dalet *et al.*, 1982).

J. Viability and characteristics of isolated hepatocytes

The simplest and most commonly used criterion for cellular integrity or viability is the trypan blue exclusion test (Berry and Friend, 1969; Green *et al.*, 1983), which is often interpreted as an indicator of cell viability. Cells with intact plasma membranes exclude dyes such as trypan blue, nigrosin and eosin, whereas damaged cells become stained, particularly intensely in the nucleus. Thus, the dye-exclusion tests detect membrane damage. The leakage of soluble enzymes such as lactate dehydrogenase has also been used as an expression of structural membrane damage. However, cells may have lesions not revealed by the trypan blue test that could lead to loss of viability at a later time. The trypan blue test, therefore, does not measure reproductive viability, but rather tests for gross structural integrity at the moment of testing (Seglen, 1976).

Intact, fresh hepatocytes are yellow in colour, have well-defined outlines and are refractile. These features easily distinguish them from the flattened, damaged cells with a "ground glass" appearance that stain darkly with trypan blue. Biochemical and morphologic studies indicate that the isolated, refractile cells are viable (Berry and Friend, 1969; Lentz and Di Luzio, 1971; Kreamer *et al.*, 1986) and have an ultrastructure similar to that of hepatocytes in organized tissue. Their only remarkable feature is the presence of microvilli all

over the cell surface that are evident in scanning electron micrographs (Seglen, 1976). Specialized membrane regions such as bile canaliculi are not evident on the surfaces of the isolated hepatocytes. The biochemical charateristics of the isolated cells remain close to those of intact liver, except that glycogen levels are low; however, net glycolysis can be measured if glucose is added to the suspension medium (Le Cam *et al.*, 1976; Seglen, 1976; Parniak and Kalant, 1985).

The hepatocyte plasma membrane is not a uniform entity (Doyle *et al.*, 1982). These cells have three major surface domains: (i) the sinusoidal surface with microvilli which constitutes 37-42% of the plasma membrane, (ii) the apposed, flat lateral surfaces which form 50-54% of the plasma membrane, and (iii) the membranes of the bile canaliculi which constitute about 6-13% of the surface. The latter two domains have no microvilli. Exchanges between the hepatocyte and the circulation take place in the sinusoidal domain of the plasma membrane. Enzyme activities differ in various membrane domains; for example, the canalicular domain is rich in γ-glutamyl transferase, while Na^+/K^+-ATPase is expressed at the sinusoidal surface. The diameter of hepatocytes from rat liver has been determined to be about 18-20 μm (Loud, 1968; Weibel *et al.*, 1969).

Uptake of solutes by hepatocytes can occur by molecular transport systems in the cell membrane, and by pinocytosis (Geuze *et al.*, 1983; Steer *et al.*, 1983). Coated pits are observed at the sinusoidal surface at the bases of, and between the microvilli. The effect of the cell membrane on the entry of materials into liver cells varies with the nature of the substance. Whereas small molecules (CO_2, urea, glycerol) enter hepatocytes by diffusion, membrane transport systems mediate the entry of various larger, biologically important solutes such as amino acids, sugars and nucleosides.

K. Fractionation of mammalian cells

Cell preparations obtained from tissues by collagenase treatment are mixtures of cell types and fractionation is required to isolate specifc cell types. Of the many techniques currently available for fractionating heterogeneous cell populations, density gradient

centrifugation has been useful in separating cells with intact membranes, from damaged cells in suspension (Brouwer *et al.*, 1984). The method is based on the original procedure of counter-streaming centrifugation described by Lindahl (1956). Modifications to this technique enabled prediction of the kinds of gradients, centrifugal forces and duration of centrifugation that would permit the isolation of of cells with known densities and diameters. This technique led to the development of isokinetic gradients (gradients in which, at constant rotor speed, a given macromolecule moves at a constant velocity) that have been widely employed for the separation of cells (Pretlow, 1971). Various techniques of gradient centrifugation, such as sedimentation at unit gravity, elutriation, isokinetic sedimentation and separation of cells by velocity sedimentation in a reorienting gradient zonal rotor, have been described (Pretlow and Pretlow, 1982).

The sedimentation rate of cells in a medium depends on the size and density of cells, and on the density and viscosity of the medium. In separating cells on density gradients the tonicity throughout the gradient must be sufficiently uniform to preclude shrinkage or swelling during the separation procedure that would alter their sedimentation behaviour. Agents such as cesium chloride and sucrose, which are used to modify medium density in the density gradient isolation of macromolecules, have high osmotic potentials in water and are unsuitable for use with cells. To avoid adverse osmotic effects, macromolecular media (Ficoll, BSA) or particulate media (Ludox, Percoll) with low osmotic potentials have been developed as density gradient material. Such media (i) do not affect cell morphology, (ii) do not permeate into cells, (iii) form low viscosity solutions, (iv) are not toxic to cells, and (v) form gradients of appropriate density for cell separation without significantly contributing to the tonicity of the medium (Pertoft and Laurent, 1982; Brouwer *et al.*, 1984). Density gradient centrifugation has, for many years, been an established method for the separation and purification of cells. Differences in density or size can be exploited to separate particles under relatively mild conditions.

L. Percoll

Percoll (Pharmacia) is a colloidal suspension of polyvinylpyrrolidone-coated silica particles, 15-20 nm in diameter. In being essentially inert in biological systems, Percoll suspensions have been used in density gradient centrifugation media for isolation of cells and organelles (Pertoft and Laurent, 1977; Pertoft *et al.*, 1978). Because of their size heterogeneity, Percoll particles sediment at different rates under centrifugation with the formation of density gradients in the range 1.0-1.3 g/ml that can be calibrated with density marker beads[3] (from Pharmacia). Density gradients achieved in Percoll suspensions are sigmoid and, by selection of initial Percoll suspension density and centrifuge rotor speed, gradients may be produced with relatively linear regions that enable resolution of cells and particle mixtures that have minor differences in density. The very low osmotic pressure of stock Percoll suspensions (1.13 g/ml; 20 mOsmoles) allows for the formation of density gradients across which osmolality differences are not significant (Vincent and Nadeau, 1984). This property of Percoll has allowed the preparation of density gradients that are essentially iso-osmotic; such gradients have been used in the isolation, from complex mixtures, of cells of high viability and intact morphology (Pertoft *et al.*, 1977; Schumaker *et al.*, 1978). Buoyant densities of cells on such gradients are often lower than those of cells from sucrose gradients where the considerable osmotic pressure of the gradient solute cause loss of water from cells. Since Percoll has low buffering capacity, pH may be adjusted in Percoll suspensions in the pH range 5.5 to 10.0. Different cell populations of rat liver cells have been successfully separated on Percoll gradients using isopycnic centrifugation (Pertoft *et al.*, 1977; Dalet *et al.*, 1982; Borreback and Osmundsen, 1983; Kreamer *et al.*, 1986). Both pre-formed gradients and self-generating gradients have been used for such preparations.

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[3] Coloured derivatives of Sephadex; each bead type is a cross-linked dextran matrix having a specific buoyant density in gradients of Percoll.

M. Isopycnic centrifugation

Isopycnic or buoyant density sedimentation refers to the centrifugal sedimentation of cells in gradients under conditions (gravitational field and time) that permit cells to reach equilibrium positions in the gradient; at their isopycnic positions, cells will be separated according to their respective densities (Pretlow and Pretlow, 1982). It is a limitation in the applicability of isopycnic centrifugation that densities of mammalian cell types are similar. Although this technique has been used widely, an alternative technique, velocity sedimentation, has proved to be superior to isopycnic sedimentation in resolution of cell mixtures because both cell diameter and density are determinants of sedimentation velocity (Hemstreet *et al.*, 1980; Jacobs *et al.*, 1981).

Density gradient centrifugation has been used to isolate and purify a variety of cell types; for example, rat spermatogenic cells (Miestrich *et al.*, 1979), rat peritoneal mast cells (Enerback and Svensson, 1980) and rat hepatocytes (Dalet *et al.*, 1982). The isopycnic separation of viable hepatocytes from other cell types derives from relationships between the density of cells and their morphological and functional integrity. Thus, the introduction of Percoll as a density determinant has led to the development of methods by which liver parenchymal cell suspensions of high viability can be prepared essentially free of other hepatic cells and cell debris (Pertoft and Laurent, 1977). A rapid and simple Percoll centrifugation procedure that avoids the use of pre-formed gradients (Kreamer *et al.*, 1986), yields suspensions of liver parenchymal cells with high viability that are free of cell aggregates, debris and non-parenchymal cells.

N. Pyrimidine nucleoside metabolism in liver

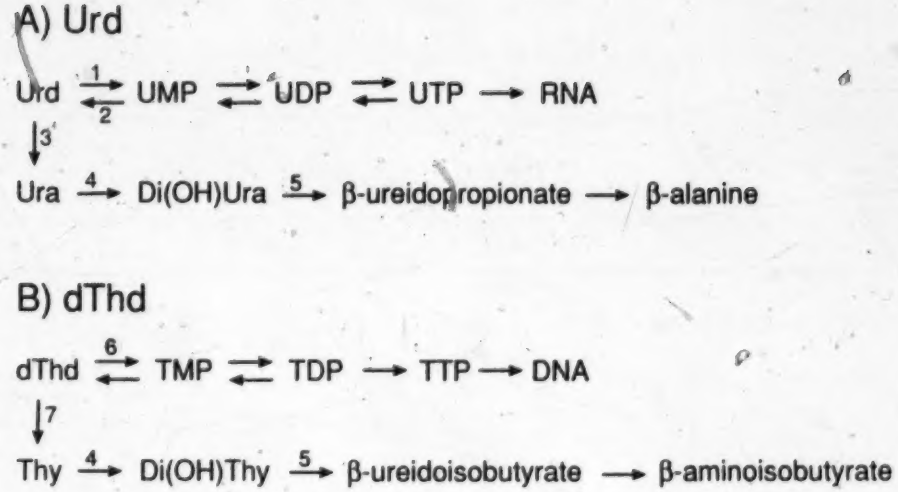
In mammalian cells, synthesis of pyrimidine nucleotides proceeds by the *de novo* pathway and by the "salvage" pathway, whereby preformed bases or nucleosides are converted into nucleotides (Levine *et al.*, 1974; Martin, 1987). The low concentrations of pyrimidine nucleosides in plasma provide nucleosides for nucleotide synthesis via the salvage pathway in many tissues (Moyer *et al.*, 1981 and 1985; Holstege *et al.*, 1984). Nucleosides in the

circulation are derived from the diet and from tissue nucleotides and polynucleotides. Fig. 2 outlines the pathways of pyrimidine nucleoside metabolism.

In mammals, the liver appears to be the primary site of uridine catabolism. In the experiments of Gasser *et al.* (1981), plasma uridine was extracted essentially completely in single passes through the liver in both intact rats and in perfused livers. *In vivo*, uridine phosphorylase appears to be primarily involved in uridine catabolism, rather than in nucleoside formation from uracil (McIvor *et al.*, 1985). Catabolism of the pyrimidine nucleosides proceeds exclusively through uracil and thymine, both of which undergo reduction via dihydropyrimidine dehydrogenase, with the formation of 5,6-dihydro derivatives. This is followed by ring opening to form ureido derivatives, which are then cleaved to β-amino acids (Martin, 1987). Dihydropyrimidine dehydrogenase (also responsible for the breakdown of 5-fluorouracil) is largely located in the liver and is rate-limiting in uracil catabolism. Of the three different cell types found in the liver, Kupffer cells demonstrate the highest rate of uridine phosphorolysis, while hepatocytes and endothelial cells, in suspension, show very little formation of uracil from labelled uridine. In addition to these findings, isolated hepatocytes were found to degrade uridine to CO_2, β-alanine and ammonia, as shown by the formation of volatile radioactivity from labelled permeant (Holstege *et al.*, 1985). These findings suggest that co-operation between the three cell types may take place in the breakdown of uridine, with emphasis on phosphorylysis in Kupffer cells and uracil catabolism in hepatocytes. Other metabolites of uridine in liver are uracil nucleotides and UDP-sugars (Yngner *et al.*, 1977). The rat has somewhat higher levels of uridine phosphorylase activity in the liver relative to other mammals (Camiener and Smith, 1965; Weber *et al.*, 1978). Comparison of the activities of uridine phosphorylase and uridine kinase in uridine metabolism in mouse tissues suggests that the phosphorylase activity correlates with the size of tissue uridine pools (Darnowski and Handschumacher, 1986).

While thymidine is rapidly phosphorylated by thymidine kinase in proliferating cells (Plagemann and Erbe, 1972; Kit, 1976), most hepatocytes from adult liver are in the G_0 phase of the cell cycle, exhibiting little DNA synthesis and only low levels of thymidine kinase

activity (Kit, 1976). Thus, while thymidine anabolism may be low in adult hepatocytes, thymidine catabolism, initiated by thymidine phosphorylase, proceeds in these cells (Woodman *et al.*, 1980). Although in adult rat hepatocytes, thymidine degradation is favoured over anabolism, following partial hepatectomy, hepatocytes in the liver remnant enter the proliferative cycle and express thymidine kinase and the capacity to anabolize thymidine. Some of the enzymes involved in the catabolism of pyrimidine nucleosides, thymidine phosphorylase, uridine phosphorylase and dihydropyrimidine dehydrogenase increase during differentiation, reaching higher levels in the adult liver than in liver of newborn rats.



1. Uridine kinase
2. 5'-Ribonucleotide phosphohydrolase
3. Uridine phosphorylase
4. Dihydropyrimidine dehydrogenase
5. Dihydropyrimidinase
6. Thymidine kinase
7. Thymidine phosphorylase

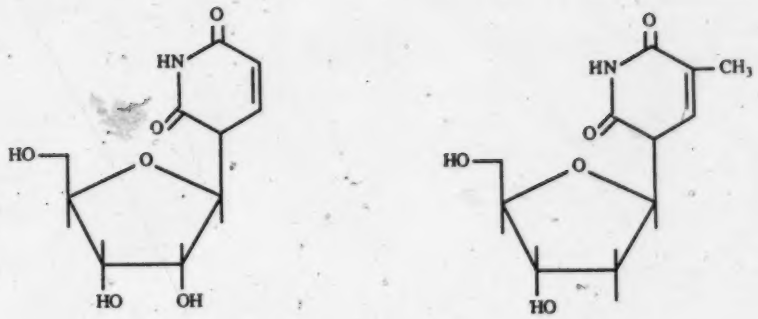
Fig. 2: Pathways of pyrimidine nucleoside metablolism in mammals.

II. INTRODUCTION TO THE PRESENT STUDY

A number of nucleoside analogues are useful anti-viral and anti-neoplastic agents and their transport across the plasma membrane is an important determinant of the metabolism of these agents (and hence of their activities) in target cells. An important aspect of current research on nucleoside drugs is centred on modulation of potency and host toxicity. This laboratory has a long-standing interest in the use of inhibitors of NT to manipulate the *in vivo* disposition of nucleoside drugs and thus to influence their toxicology. Understanding the transport of physiological nucleosides into cells is fundamental to understanding the transport and subsequent metabolism of the nucleoside analogs used in chemotherapy, since the NT systems are major routes of entry of the unphysiological nucleosides into cells (Paterson and Cass, 1986).

The transport of thymidine into isolated rat hepatocytes by a concentrative and energy-dependent process was shown by Ungemach and Hegner (1978). In this laboratory, it was shown that NBMPR inhibited cytidine uptake in perfused mouse livers suggesting the operation of a transporter-mediated process for nucleoside entry into liver cells (Kolassa and Paterson, 1982). Plagemann and Wohlhueter (1985) have reported the presence of high-affinity (K_D, 0.43 nM) NBMPR binding sites in isolated rat hepatocytes. In those experiments, transport of uridine by the hepatocytes showed high sensitivity to NMBPR inhibition ($IC_{50} < 10$ nM).

NT systems in several lines of cultured rat hepatoma cell lines show low sensitivity ($IC_{50} > 1$ μM) to NBMPR (Paterson *et al.*, 1987). The present study was undertaken to characterize the NT system(s) in hepatocytes. Two physiological pyrimidine nucleosides, uridine and thymidine (Fig. 3) were employed in the study.



Urd dThd

Fig. 3: Structural formulae of uridine and thymidine.

III. MATERIALS AND METHODS

A. Materials

Chemicals

Nitrobenzylthioinosine was prepared in this laboratory by an established method (Noel and Robins, 1962; Paul *et al.*, 1975). The starting material, 6-thioinosine, was a gift from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Uridine and thymidine were purchased from Sigma Chemical Co., St. Louis, MO; collagenase (from *Clostridium histolyticum*) was from Sigma (Type IV) and from Boerhinger Mannheim Canada, Dorval, Que.; Percoll from Pharmacia Canada, Dorval, Que., and PEI-cellulose plates (Polygram 300 PEI/UV_{254}) were from Brinkman Instruments, Westbury, NY. Nylon macrofilters (60 μm mesh size) were obtained from Cole-Parmer, Chicago, IL. Eagle's minimum essential medium for suspension culture (S-MEM), in powdered form, was purchased from Gibco Laboratories, Burlington, Ont. Alamine 336 was from Henkel Co., Kankakee, IL., and Freon TF was from Du Pont Canada Inc., Maitland Ont.

Radiochemicals

^3H_2O (100 mCi/ml) was purchased from ICN, Irvine, CA; [1,2-^{3}H]polyethylene glycol (2 mCi/g) from New England Nuclear, Boston, MA; [5-^{3}H]uridine (20 Ci/mmol), [methyl-^{3}H]thymidine (60 Ci/mmol) and [G-^{3}H]NBMPR (23 Ci/mmol) were from Moravek Biochemicals Inc., Brea, CA. All radiochemicals were purified by HPLC to greater than 98% radiochemical purity using C_{18} μ Magnum 9 column (Whatman Int., Clifton, NJ) eluted with methanol/water solutions.

Animals

Male Sprague-Dawley rats weighing 200-250 g (Health Sciences Animal Services, University of Alberta) were maintained at 22 °C with alternate 12-hr light and dark cycles,

and fed rodent laboratory chow *ad libitum*.

B. Methods

Media

The composition of cell suspension media employed in the preparation of hepatocytes and transport experiments is summarized in Table 1; media names (left column) are used throughout the text. Two additional media based on a cell culture medium, S-MEM (Eagle's minimum essential medium for suspension culture), were also used: (i) MEM growth medium [S-MEM medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and calf serum (10%)] was employed in the preparation of hepatocytes by the Percoll density gradient isolation procedure, and (ii) MEM transport medium [S-MEM medium without $NaHCO_3$ and containing 20 mM HEPES (pH 7.4, 22 °C)] was employed in experiments that evaluated NBMPR inhibition of uridine fluxes in hepatocytes.

Isolation of hepatocytes

Hepatocytes from male Sprague-Dawley rats were isolated by the following collagenase perfusion method, which is essentially that descibed by Berry and Friend (1969), and by Seglen (1976).

(a) *Liver perfusion*: After overnight deprivation of food, male Sprague-Dawley rats weighing 200-250 g, were injected intraperitoneally with 0.2 ml heparin sulphate solution (1000 U total) 30 min prior to ether anesthesia, after which the peritoneal cavity was opened by a transverse incision across the lower abdomen and a longitudinal incision up to and beyond the sternum. The viscera were moved to the left side of the animal to expose the inferior *vena cava* and the portal vein. Ligatures were passed around the portal vein, at 2 and 4 cm from its junction with the liver capsule and were loosely tied. The portal vein was then transected between the ligatures and the caudal ligature tied.

Table 1. Composition of media.

Medium	Final concentration (mM)									
	NaCl	KCl	Phosphate (pH 7.4)[1]	$MgCl_2$	$MgSO_4$	$CaCl_2$	HEPES (pH 7.4)	D-Glucose	NMG^+	$EDTA^2$
1. EDTA	140	5	1		0.8		10			0.5
2. Colla-genase[3]	84	7		0.5		5	10			
3. HBS	141	7				1	10			
4. Na^+	140		10	0.5		1		6		
5. K^+		140	10	0.5		1		6		
6. NMG^+			10	0.5		1		6	140	
7. PBS	140	3	10	0.5		1				

[1] Sodium salts in medium no. 4, sodium and potassium salts in medium no.7 (Dulbecco and Vogt, 1974), and potassium salts in other media; pH values were adjusted at 22 °C.

[2] Disodium salt.

[3] Concentration, 0.5 g/l.

The upper portion of the portal vein was cannulated with 0.76 mm I.D. polyethylene tubing (PE-60 Intramedic tubing, Clay Adams, Parsippany, N.J.), and the tubing was then secured by tying the remaining ligature.

Perfusion with EDTA medium was started at a flow rate of about 20 ml/min and the inferior *vena cava* was severed immediately to permit free flow of the perfusion medium. After increasing the flow rate to about 40 ml/min, perfusion was continued until most of the blood had been flushed from the liver, judging by blanching of the liver colour. This required about 250-300 ml of EDTA medium and took 8-10 min. Respiration was then ended by puncturing the diaphragm and the perfusion continued with collagenase medium until the liver became swollen and fluid was seen to collect under the capsule. This required about 100-150 ml of medium and took about 5 min. The perfusion was done without recirculation of medium. The cannula was then disconnected and the liver excised from the carcass and kept on ice in HBS medium. During perfusion, lobes of the liver were in their natural positions to permit medium flow to all parts of the liver.

(b) *Dispersion of cells from collagenase-treated liver*: The perfused liver was placed in a petri dish containing ice-cold HBS medium, and extraneous tissue was cut away from the liver. While the liver was held with forceps in the portal vein region, the capsule was torn with forceps and cells were freed into the medium by shaking the liver in the medium. After transferring capsule remnants into fresh HBS medium, additional cells were shaken free. The contents of the two dishes were then combined and the suspension was filtered through nylon mesh (60 μm mesh size). The total volume of the cell suspension was about 75 ml. After the suspension was centrifuged at 65 x g for 3 min in polycarbonate tubes, cells were resuspended in 50 ml MEM. Cell clumps were dispersed with gentle pipetting and the suspension was centrifuged at 25 x g for 5 min. Cells were resuspended in 50 ml of MEM and cell concentration was determined with an electronic cell counter prior to Percoll gradient purification.

Percoll gradient isolation of intact hepatocytes

(a) *Preparation of iso-osmotic Percoll suspension*: Iso-osmotic Percoll suspension was prepared by the procedure described by Dalet *et al.* (1982) and by Kreamer *et al.* (1986) with slight modifications. A Percoll suspension in MEM was prepared by adding 38 ml of primary stock Percoll suspension to 62 ml S-MEM solution (without antibiotics and serum). The pH of the suspension was adjusted to 7.4 at 22 °C by aeration (about 45 min) with 5% CO_2 in oxygen, and calf serum added to a final concentration of 10%. The density of this solution was 1.06 g/ml.

(b) *Hepatocyte isolation*: Suspensions of freshly isolated hepatocytes in MEM were mixed with 38% Percoll-MEM suspension to achieve cell concentrations of 1 x 10^6 cells/ml. Portions of the hepatocyte-Percoll suspensions (35 ml) in 50-ml polycarbonate tubes were spun in a Sorvall RC-5 centrifuge (SS 34 rotor) at 20,000 x g for 20 min at 4 °C. Each hepatocyte isolation operation included a portion of the Percoll-MEM suspension containing density marker beads in place of hepatocytes, in order to calibrate the Percoll gradient. After centrifugation, gradient fractions containing non-viable cells and cell debris were removed by aspiration and the bands containing viable cells were pooled and resuspended in MEM. Cells were washed twice with MEM medium by centrifugation (25 x g, 3 min, 4 °C) to separate residual Percoll from cells, and the cells suspended in MEM to a final density of 2 x 10^5 cells/ml.

(c) *Cell viability*: "Viability" of the Percoll-isolated hepatocytes was assayed by determining the fraction of the cell population that excluded trypan blue. Equal volumes (100 μl) of cell suspension and trypan blue solution (0.4 g trypan blue in 100 ml 0.15 M NaCl solution) were mixed and, after 5 min, the proportion of dye-excluding cells was determined using a hemocytometer counting chamber. About 500 cells were enumerated.

NBMPR Binding

The site-specific, equilibrium binding of NBMPR to hepatocytes was measured by a previously described method in which cells were equilibrated with [^{3}H]NBMPR and recovered

by centrifugal pelleting under oil (Paterson *et al.*, 1980). Freshly isolated cells were incubated with graded concentrations of [^{3}H]NBMPR in the presence or absence of 20 μM NBMPR. In each assay, duplicate incubation mixtures were prepared by layering 100 μl portions of graded concentrations of [^{3}H]NBMPR in PBS over 100 μl of silicone/paraffin oil solution contained in 1.5-ml polypropylene microcentrifuge tubes. The oil solution, specific gravity 1.02, was prepared by mixing 400 ml silicone 550 oil (Dow Corning, Mississauga, Ont.) and 100 ml light paraffin oil (Saybolt viscosity 125-135, Fisher Scientific, Fair Lawn, N.J.). Portions (300 μl) of NBMPR (20 μM, final) in PBS (non-specific binding) or PBS (specific binding) were then added to the tubes. Incubation mixtures were completed by the addition of 10^6 hepatocytes in PBS to make the final volume of 1.0 ml. Mixtures were then incubated at 22 °C for 20 min to allow equilibration of [^{3}H]ligand with cellular binding sites. After incubation, cells were centrifugally pelleted (16,000 x g, 30 sec) under the oil layer and 100 μl portions of the supernatant fractions assayed for ^{3}H-activity to estimate concentrations of free [^{3}H]NBMPR (Kolassa and Paterson, 1982). Tubes were then rinsed twice above the oil with water. After the second rinse, oil and water were removed by aspiration and 0.5 ml of 5% Triton X-100 was added to each tube to solubilize cell pellets. The tubes with their contents in place were then placed in scintillation vials containing 8 ml of xylene-Triton X-100 scintillant (Pande, 1976); vial contents were then mixed vigorously and the vials were assayed for ^{3}H-activity.

Nucleoside fluxes

Nucleoside fluxes in isolated hepatocytes were assayed at 22 °C within 1 hr after cells were removed from the Percoll gradient. The effects of experimental variables (such as permeant concentration, ionic composition of medium or presence of NT inhibitors) on nucleoside fluxes in hepatocytes were measured in experiments that employed replicate assay mixtures in which hepatocytes were exposed for graded intervals to [^{3}H]permeants in order to determine progress curves (time courses) for cellular uptake of permeant. Parabolas were fitted to time course data by a computer program (Harley *et al.*, 1982) that yielded the slopes of the progress curves at time zero (the coefficients of the first-order term in the parabolas).

Slopes at time zero of the time courses of permeant uptake by cells measure unidirectional, inward fluxes of permeant.

In these experiments, flux assays were conducted with sets of replicate assay mixtures assembled in microcentrifuge tubes placed in a centrifuge rotor (Eppendorf model 5412), to start intervals of permeant uptake. Assay mixtures were completed by the manual addition of cells in response to metronome signals, or in several experiments by addition of [^{3}H]permeant by means of an automatic pipettor (Micromedic Digiflex automated pipettor, model 33010, ICN Biomedicals Inc., Costa Mesa, CA). Intervals of permeant uptake were ended by centrifugal pelleting of cells under oil, allowing 2 sec after centrifuge switch-on for clearance of cells from the incubation mixture. In the preparation of assay mixtures, 100 µl portions of (i) [^{3}H]permeant-containing medium, or (ii) cell suspension (2×10^5 cells) in experiments with automated pipetting, were layered over 100 µl silicone/paraffin oil solution in 1.5-ml microcentrifuge tubes placed in a centrifuge rotor. Intervals of nucleoside uptake were started by the manual addition of 100 µl cell suspension, or by the automated addition of [^{3}H]permeant and ended by pelleting cells under oil. For assays of nucleoside flux in the presence of NBMPR, cells were incubated with the inhibitor for 10 min before assays began.

Zero-time uptake: In the manual assays, cell-associated [^{3}H]permeant at zero time was determined in the cold. Assays were performed at 0-4 °C in the presence of excess unlabelled permeant by adding cell suspension to tubes containing [^{3}H]permeant and unlabelled permeant (1 mM, final concentration) and pelleting cells immediately.

Intracellular water space: The water space in cell pellets was determined by using ^3H_2O in place of nucleoside permeant in the uptake assay and the extracellular space determined by substituting [^{3}H]PEG for permeant.

Metabolism of pyrimidine nucleosides

To assess the extent of metabolism of uridine and thymidine by hepatocytes in suspension, acid-soluble cell extracts were prepared and analyzed by thin-layer chromatography. Assay mixtures were assembled as follows in 1.5-ml microcentrifuge tubes

containing 100 µl of 0.4 M trichloroacetic acid (TCA) under 400 µl of silicone/paraffin oil: (i) 200 µl portions of 100 µM [^{3}H]permeant in Na^+ medium were placed on the oil layer, (ii) graded intervals of permeant uptake were begun by adding 200 µl of cell suspension (4 x 10^5 cells in Na^+ medium) and ended by pelleting cells through the oil into the TCA layer, (iii) after aspiration of the medium fraction, tubes were kept in ice-water for 1 hr. Tube walls above the oil were washed twice and, after aspiration of the oil, 90 µl portions of the TCA extracts were mixed with equal volumes of alamine/freon solution (25 ml alamine 336 and 75 ml Freon TF) (Khym, 1975) in microcentrifuge tubes, and (iv) after centrifuging the latter mixtures, the supernatant fractions were stored at -20 °C for TLC analysis.

Thin-layer chromatographic analysis of TCA cell extracts

PEI-cellulose TLC sheets were washed in distilled water overnight and air-dried prior to use (Bols *et al.* 1980). Ten nmol portions of marker compounds, mixed with 10 µl portions of neutralized cell extracts, were spotted on PEI-cellulose TLC sheets with drying at room temperature between applications. The chromatograms were developed by upward migration of distilled water to 16 cm above the origin. Marker compounds were located on the dried chromatograms under UV light and 5 mm sections of chromatogram lanes were placed in scintillation vials and incubated overnight with 1 ml of 0.5 M NaOH. After adding 0.1 ml of concentrated HCl and 8 ml of scintillation fluid, these samples were assayed for radioactivity by liquid scintillation counting.

IV. RESULTS

A. Isolation of hepatocytes

The preparation of hepatocytes by the two-step collagenase perfusion method was originally described by Berry and Friend (1969) and later modified by Seglen (1976). The version of this method employed in the present study involved (i) initial perfusion with medium containing 0.5 mM EDTA, followed by (ii) perfusion with medium containing collagenase and 5 mM Ca^{2+}. This procedure yielded about 2 x 10^8 viable hepatocytes per adult rat liver. The separation of viable cells from non-viable cells on a Percoll gradient is illustrated in Plate 1. The lower band (A) consisted almost entirely of viable cells which had buoyant densities between 1.076 and 1.087 g/ml. Densities were estimated from the banding of density marker beads in a gradient tube that lacked cells, prepared with each set of gradient tubes. The upper band (B) of cell material on the gradient consisted of damaged cells that were permeable to trypan blue. Cells in that layer had buoyant densities of about 1.047 g/ml. Plate 2, a photomicrograph of an unfractionated preparation of hepatocytes, shows that about half of the cells were permeable to trypan blue. Stained cells had a "ground-glass" appearance and nuclei were stained intensely. Cell debris and nuclei from broken cells are apparent in the photomicrograph. The Percoll-purified preparation shown in Plate 3 was typical; cells in these preparations were almost entirely viable hepatocytes with little debris and few damaged cells present. Single cells were the most abundant, but aggregates of 2 to 4 cells were also present in these preparations. The viable cells were rounded, refractile and yellowish-brown in colour. Table 2 summarizes yields and fractional viability of band A cells in a group of typical preparations from adult male rats in this study.

Plate 1: Separation of viable and non-viable hepatocytes on self-generating Percoll gradients. Cell suspension was mixed with Percoll suspension in polycarbonate tubes and centrifuged at 20,000 x g for 20 min as described in Materials and Methods. Band A contained viable hepatocytes and band B contained damaged cells and debris. The tube on the right contained density marker beads in place of hepatocytes.

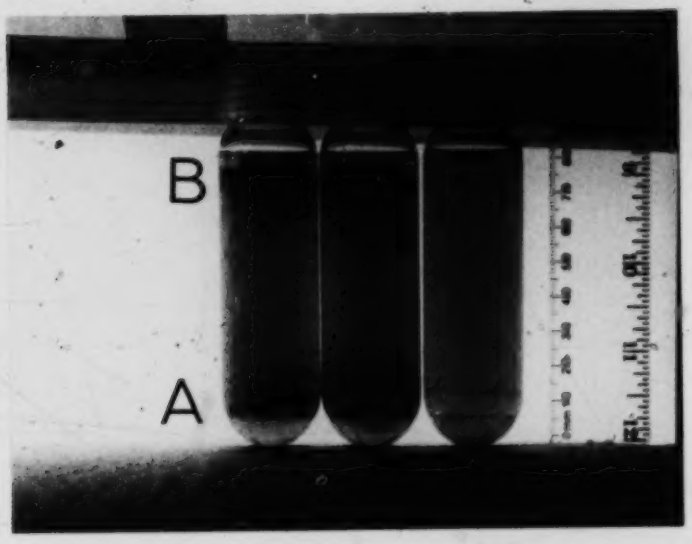


Plate 2: Photomicrograph of crude hepatocyte preparation. Unfractionated cell suspension was mixed with an equal volume of 0.4% trypan blue solution, as described in Materials and Methods. Nuclei of damaged cells are stained with the dye. Viable cells are yellowish-brown (160 x magnification).

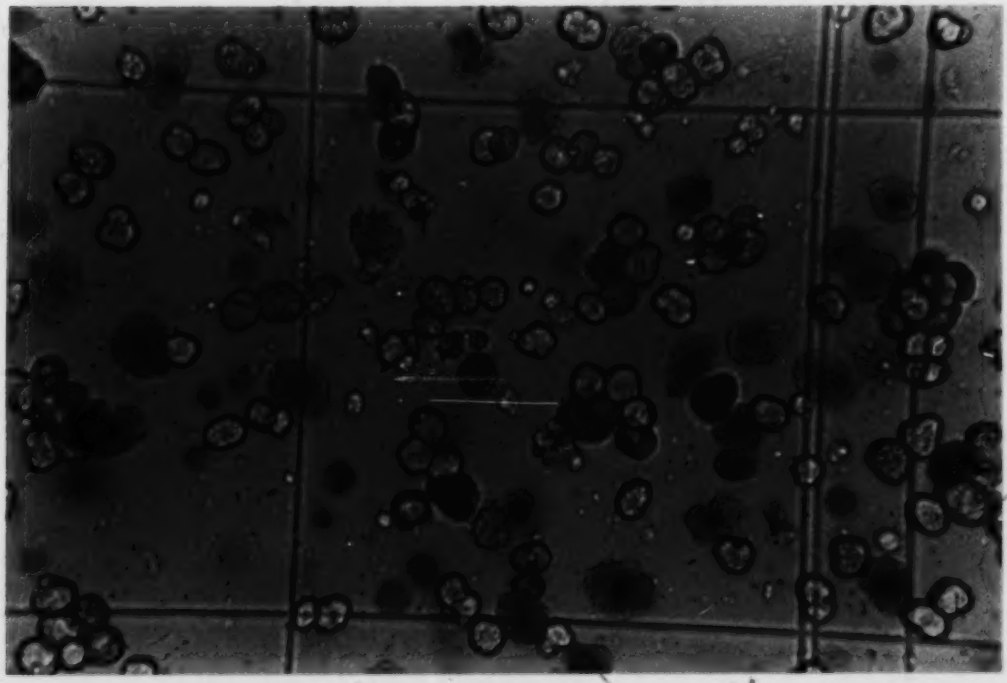


Plate 3: Photomicrograph of Percoll-purified hepatocyte preparation. Cell suspension was mixed with an equal volume of 0.4% trypan blue solution, as described in Materials and Methods. Viable cells are rounded and refractile, and damged cells are stained (160 x magnification).

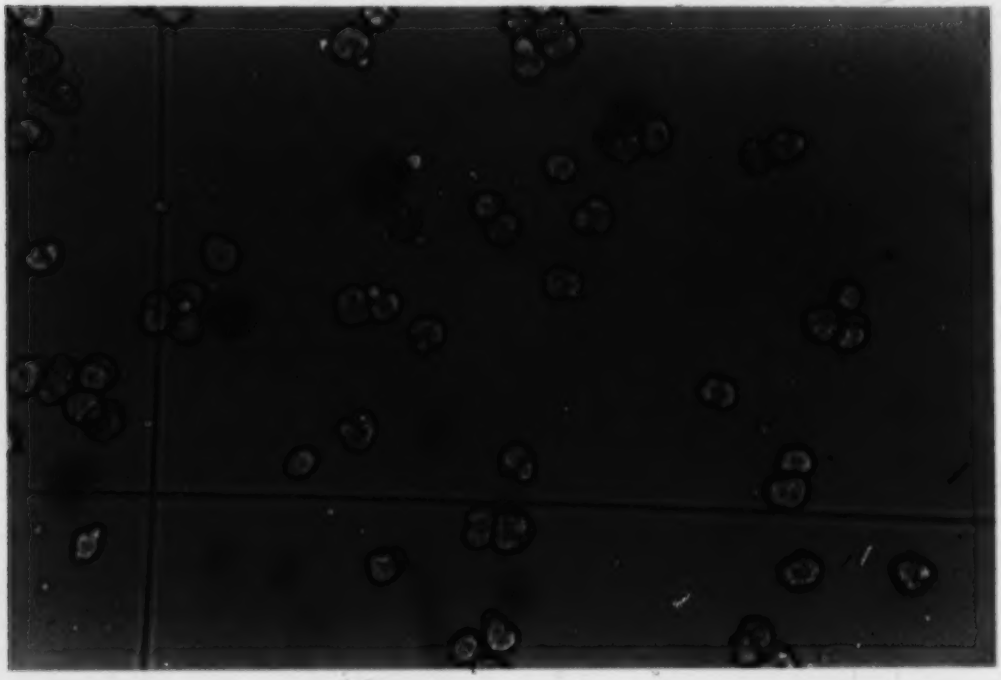


Table 2. Yield of viable hepatocytes from the livers of male rats weighing 200 - 250 g. After perfusion of livers *in situ* with collagenase solution and release of hepatocytes from the excised organ into suspension, viable cells were isolated by Percoll-gradient centrifugation. The values shown are means (± S.D) from 15 different hepatocyte preparations.

Unfractionated preparation ($\times 10^6$)	Percoll fraction A (viable cells)		Fractional viability (dye-excluding cells, %)
	($\times 10^6$)	(% total)	
358 ± 35	184 ± 34	52 ± 9	95 ± 2

B. The presence of high-affinity NBMPR binding sites on hepatocytes

To detect the presence of sites with high affinity for NBMPR, hepatocytes were equilibrated with graded concentrations of [^{3}H]NBMPR in the absence or presence of 20 μM non-isotopic NBMPR. The difference between the cell content of [^{3}H]NBMPR at equilibrium (a) in the presence, and (b) absence of 20 μM non-isotopic NBMPR, is defined as site-bound NBMPR, or as specifically bound NBMPR. The saturability of site-specific binding of [^{3}H]NBMPR to hepatocytes was demonstrated by the experiment depicted in Fig. 4. NBMPR binding data obtained in this way were subjected to mass law analysis by the method of Scatchard using the "Ligand" computer program (Munson and Rodbard, 1980) to obtain plots such as that shown in Fig. 5. In these analyses, straight lines appeared to fit the data, suggesting that NBMPR binding sites on hepatocytes are of a single type. Binding constants from these analyses are presented in Table 3. K_D and B_{max} values for site-bound NBMPR compare reasonably with those found for other cell types. Human erythrocytes have about 10^4 binding sites per cell (Pickard *et al.*, 1973; Cass *et al.*, 1974), whereas S49 mouse lymphoma cells (Cass *et al.*, 1981), Novikoff UA hepatoma cells (Paterson *et al.*, 1987) and HeLa S3 cells (Dahlig-Harley *et al.*, 1981), have about 10^5 binding sites per cell. IEC-6 rat intestinal epithelial cells have about 10^6 sites per cell (Jakobs and Paterson, 1986). K_D values for NBMPR dissociation from the site-ligand complex in cells of the above-mentioned types range between 0.1 and 1.4 nM.

C. Intracellular water space

The water content of freshly isolated hepatocytes was determined as the difference between the ^3H_2O space and the [^{3}H]PEG space in cell pellets obtained after centrifugal pelleting of hepatocytes under oil. As shown in Table 4, the cell water space in hepatocyte pellets after suspension in Na^+ medium was 4.5 $\pm$ 0.6 μl/10^6 cells (n=11) and the extracellular space was 20.8 $\pm$ 2.5% of the pellet water space.

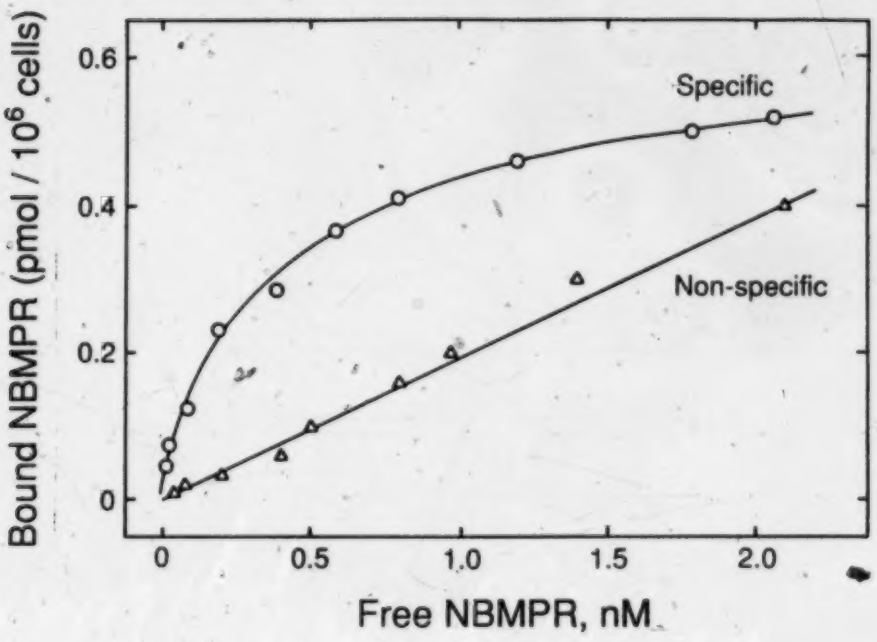


Fig. 4: Presence of NBMPR binding sites on rat hepatocytes. Percoll-isolated hepatocytes in replicate incubation mixtures were exposed for 20 min at 22 °C to graded concentrations of [^{3}H]NBMPR in PBS medium with and without 20 μM non-isotopic NBMPR. The ^{3}H content of medium and cells was determined as described in Materials and Methods. Site-specific binding of [^{3}H]NBMPR (○) was determined as the difference between cell-associated [^{3}H]NBMPR in the absence of NBMPR (data not shown) and in the presence of 20 μM non-isotopic NBMPR (△). A mass law analysis of these data is shown in Fig. 5. These data (means of duplicate determinations) are typical of results obtained in 3 similar experiments.

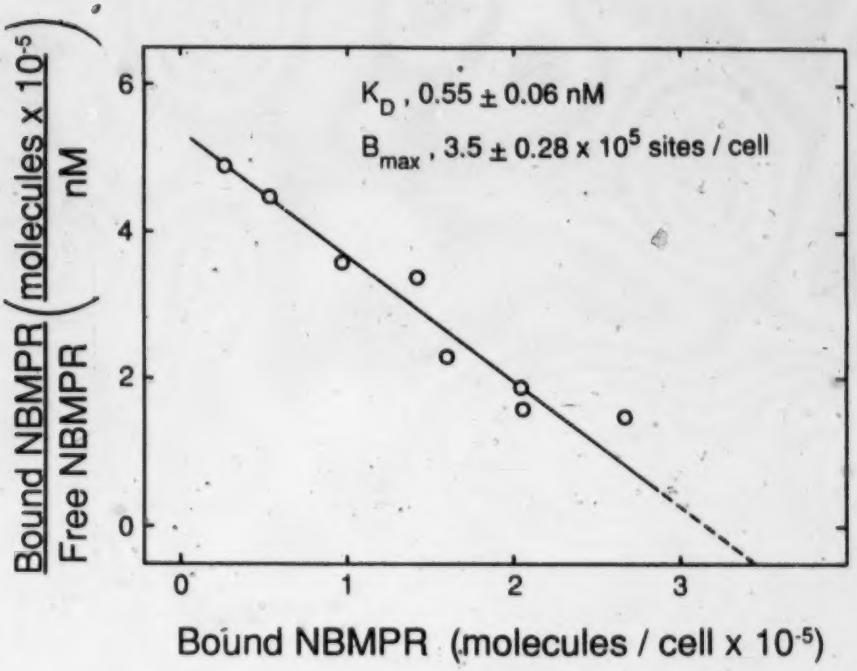


Fig. 5: Scatchard plot of data from Fig. 4. Computer-assisted mass law analysis of binding data using the "Ligand" program (Munson and Rodbard, 1980) yielded the binding constants shown.

Table 3. Site-specific, equilibrium binding of NBMPR to rat hepatocytes. Binding constants (K_D, B_{max}) were determined by computer-assisted mass law analysis of binding data using the "Ligand" program. Values shown are means (± S.D).

Expt.	K_D (nM)	B_{max} (sites/cell x 10^{-5})
1	0.80 ± 0.22	7.18 ± 1.22
2	0.25 ± 0.04	2.07 ± 0.14
3	0.55 ± 0.06	3.50 ± 0.28
Mean	0.53 ± 0.08	4.27 ± 0.42

Table 4. Water space in hepatocytes suspended in different media. Cellular water space was determined as the difference between ^3H_2O space and [^{3}H]PEG space in oil layer pellets of hepatocytes. Values reported are means (± S.D.).

Medium	Cellular water space (μl/10^6 cells)
Na^+	4.5 ± 0.6 (n = 11)
K^+	3.9 ± 0.4 (n = 3)
NMG^+	4.2 ± 0.8 (n = 5)
MEM	4.3 ± 1.2 (n = 4)

Cellular water space in hepatocytes suspended in Na^+, K^+, NMG^+ media, or in MEM transport medium, did not differ significantly, and the values reported here (Table 4) are in general agreement with values reported by others who employed [^{3}H]inulin in determining the extracellular space (Ungemach and Hegner, 1978; Plagemann and Wohlhueter, 1985).

D. Inhibition of uridine transport by NBMPR

The NBMPR sensitivity of inward fluxes of uridine in rat hepatocytes was evaluated in experiments that measured time courses of uridine uptake by the hepatocytes over short intervals (0 - 30 sec). Brief intervals of exposure to uridine were employed in order that initial rates of uridine uptake by the cells were defined by the time courses. Initial rates of permeant uptake by cells measure unidirectional, inward fluxes of permeant and, therefore, define rates of inward transport of that permeant (Paterson and Cass, 1986). In these experiments, cell suspensions were incubated in MEM transport medium in the presence and absence of 1 μM NBMPR. In the experiment of Fig. 6, replicate assay mixtures containing hepatocytes in MEM transport medium with 100 μM [^{3}H]uridine were incubated at 22 °C for the intervals specified (which include a 2-sec interval for cell pelleting). These intervals were begun by addition of cell suspension and ended by centrifugal pelleting of the cells under oil. The cell content of [^{3}H]uridine in the absence of NMBPR exceeded the extracellular concentration of uridine after 15 sec of incubation, but a steady state cell content of the permeant had not been reached at that time. In the presence of 1 μM NBMPR, the cell content of uridine achieved during this interval was about half of that achieved in the absence of NBMPR. The initial rate of uridine (100 μM) uptake in the presence of NBMPR was about 65% of that in the absence of the inhibitor. A substantial NBMPR-insensitive component of uridine uptake was evident in this experiment.

The effects of graded concentrations of NBMPR on inward fluxes of uridine in hepatocytes were evaluated using the initial rate method. Initial rates of uridine uptake were determined from the coefficients of the first order term in parabolas fitted to uptake data by polynomial regression analysis using a computer program (HP-41C Stat Pac,

Hewlett-Packard). Measured in this way, fluxes of [^{3}H]uridine (20 μM, final) in hepatocytes exposed to NBMPR for 10 min at 22 °C, are plotted in Fig. 7 as percentages of control rate (influx rate measured in the absence of NBMPR) against inhibitor concentrations. The relationship between flux and NBMPR concentration is biphasic (Fig. 7) with about 45% of transport activity remaining when high-affinity binding sites were occupied by NBMPR. The IC_{50} value for the NBMPR-sensitive component of [^{3}H]uridine uptake was about 0.5 nM. Higher NBMPR concentrations inhibited uridine transport with an IC_{50} value greater than 10 μM. These results show that a component of uridine permeation in hepatocytes is of high sensitivity to NBMPR, while the remaining process(es) of uridine entry are of low sensitivity to the inhibitor.

Recent reports have shown the presence of sodium-dependent NT systems in a number of mammalian cell types, including epithelial cells and leukemic cells (Le Hir and Dubach, 1984; Spector and Huntoon, 1984 and 1985; Darnowski and Handschumacher, 1986; Jakobs and Paterson, 1986; Darnowski *et al.*, 1987; Dagnino *et al.*, 1987). The sodium-linked NT systems described above are of low sensitivity to NBMPR. Johnston and Geiger (1989) have reported that the Na^+-linked transport of adenosine in dissociated brain cells from rat was inhibited by NBMPR. The possibility that a component of uridine permeation in hepatocytes was sodium-linked, was explored by comparing uridine permeation rates in Na^+ transport medium with those in medium in which K^+ replaced Na^+ (Fig. 8). In Na^+ medium, uridine levels in hepatocytes were double the extracellular concentration within 30 sec, whereas in K^+ medium, uridine levels only approached extracellular levels during the same interval. Initial rates of uridine uptake in Na^+ medium were about twice those in Na^+-free medium. Similar reductions in uridine flux resulting from the substitution of K^+ for Na^+ were seen in two other similar experiments. The experiment of Fig. 9 showed that uridine fluxes in hepatocytes in K^+ medium were insensitive to NBMPR. Table 5 compares uridine fluxes in Na^+ medium and K^+ medium with and without NBMPR.

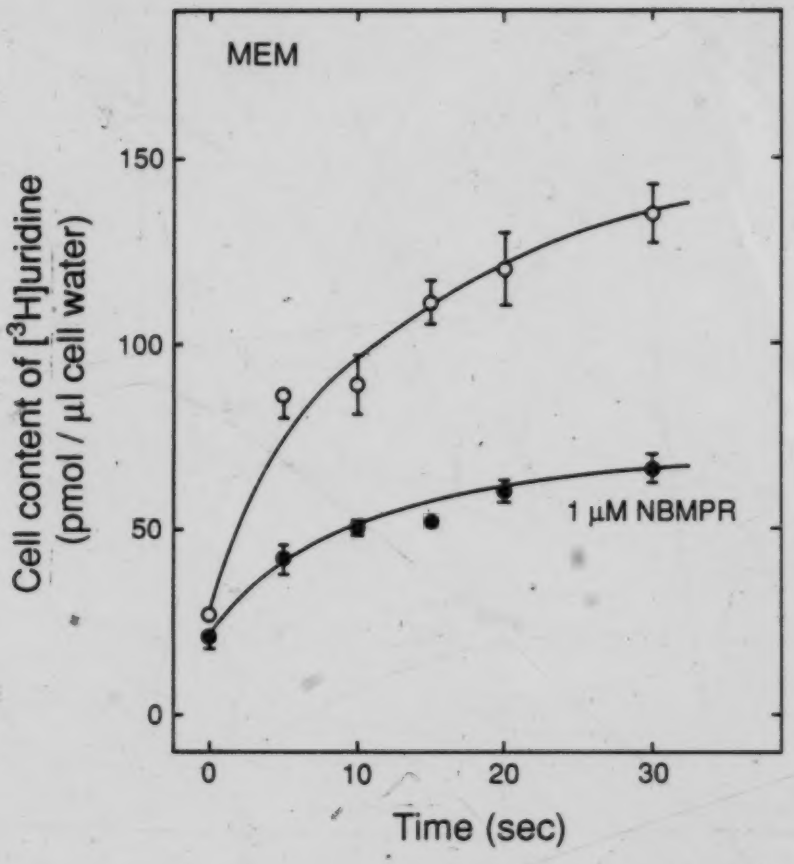


Fig. 6: NBMPR inhibition of uridine transport by hepatocytes. Percoll-isolated hepatocytes were exposed for the time intervals specified to MEM transport medium containing 100 μM [^{3}H]uridine with (●) and without (○) 1 μM NBMPR. Replicate assay mixtures contained 2 x 10^5 cells in a final volume of 200 μl. Cell-associated ^{3}H-activity was determined as described in Materials and Methods. Data shown are means (± average deviations from the mean) of duplicate assays. These results were typical of 2 other similar experiments.

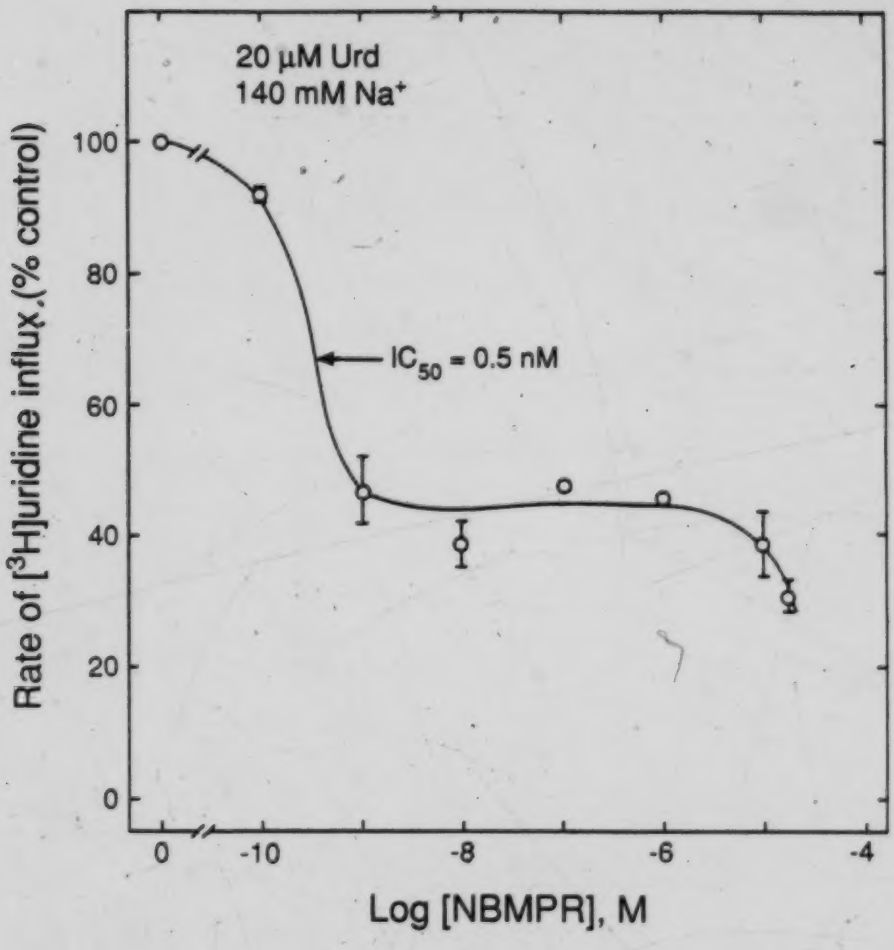


Fig. 7: NBMPR inhibition of uridine transport by hepatocytes. Replicate assay mixtures containing 2 x 10^5 cells in 100 μl Na^+ medium with and without NBMPR (control) at twice the indicated concentrations were incubated for 10 min at 22 °C before addition of 100 μl of medium containing [^{3}H]uridine (20 μM, final) to initiate intervals of uridine uptake. Inward fluxes of [^{3}H]uridine were assayed as described in Materials and Methods. The control rate of uridine influx was 2.0 pmol/μl cell water/sec. The data are means (± S.D.) of triplicate determinations.

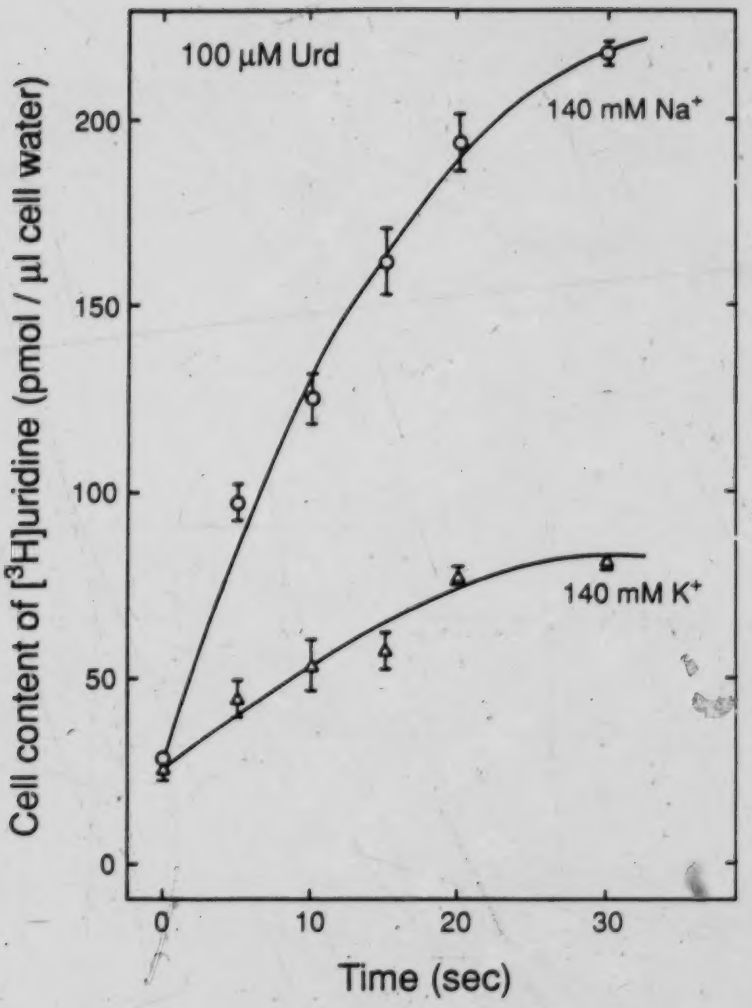


Fig. 8: Na^+-dependence of uridine transport in rat hepatocytes. When completed, the replicate assay mixtures contained 2 x 10^5 hepatocytes in 200 μl medium with 100 μM [^{3}H]uridine. To assay [^{3}H]uridine uptake by hepatocytes, the specified intervals of uptake at 22 °C were begun by addition of cell suspension (to complete assay mixtures) and ended by centrifugal pelleting under oil, as described in Materials and Methods. Na^+ medium (○) contained 140 mM Na^+, and K^+ medium (△) contained 140 mM K^+ in place of Na^+. Error bars represent average deviations from the means (plotted) of duplicate assays. The results of this experiment were typical of 2 other similar experiments.

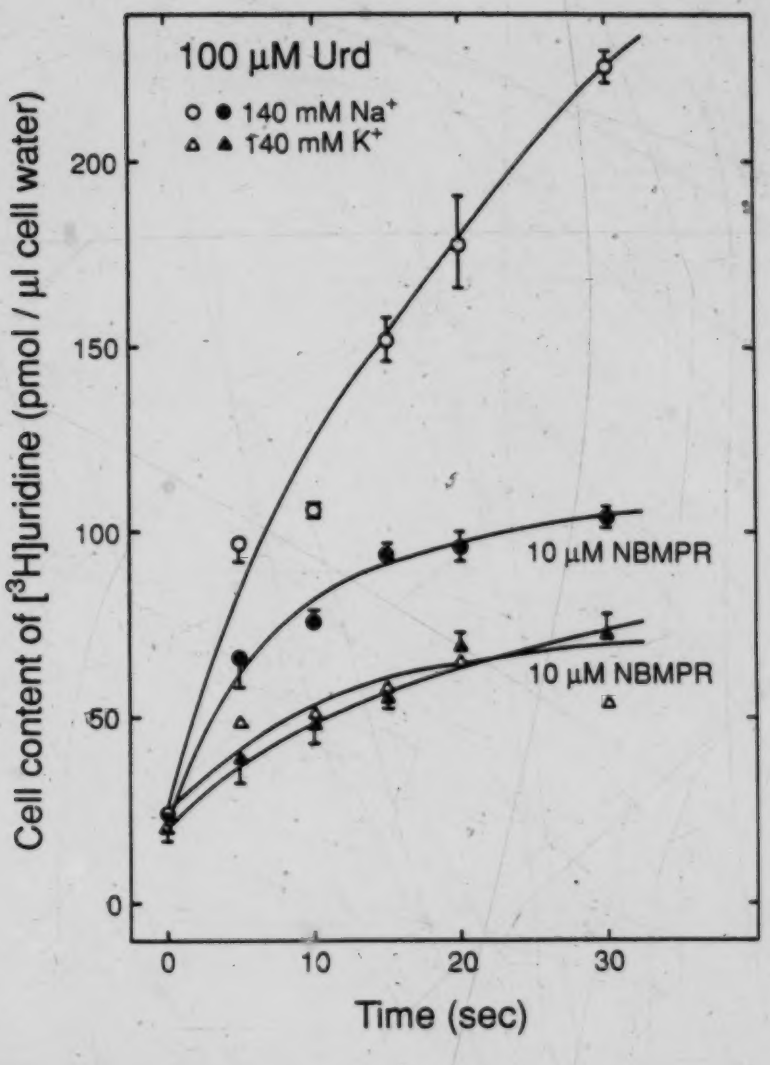


Fig. 9: NBMPR inhibition of Na^+-dependent uridine transport by hepatocytes. Completed assay mixtures contained 2 x 10^5 hepatocytes in 200 µl Na^+ medium or K^+ medium with 100 µM [^{3}H]uridine. Cells were exposed to [^{3}H]uridine at 22 °C for the intervals specified, as in the experiment of Fig. 8. Na^+ medium and K^+ medium contained 140 mM Na^+ (○, ●) and 140 mM K^+ (△, ▲), with (●, ▲) and without (○, △) 10 µM NBMPR. Error bars indicate average deviations from the means (plotted) of duplicate assays. Two additional similar experiments yielded similar results.

Table 5. Inward fluxes of uridine in hepatocytes. Initial rates of uptake of 100 μM [^{3}H]uridine by hepatocytes were compared in the presence and absence of NBMPR. Values listed are means (± S.E.M.) from 3 experiments.

	Uridine flux (pmol/μl cell water/sec)		
Transport medium	With NBMPR	Without NBMPR	Student's *t*-test
Na^+	4.5 ± 0.8[1]	7.1 ± 1.4	($p < 0.05$)
K^+	4.4 ± 0.5[1]	4.2 ± 0.4	(N.S.)
MEM	2.5 ± 0.2[2]	4.8 ± 0.4	($p < 0.05$)

[1] 10 μM NBMPR

[2] 1 μM NBMPR

N.S. = not significant

E. The metabolism of uridine in hepatocytes

In the rat, uridine from the circulation is rapidly metabolized by the liver (Gasser *et al.*, 1981; Moyer *et al.*, 1981). The first step in the catabolism of uridine in the liver, the formation of uracil, occurs predominantly in the Kuppfer cells (Holstege *et al.*, 1985). The extent of uridine catabolism in isolated hepatocytes was assessed by (i) incubating cells with 50 μM [^{3}H]uridine for graded intervals, and (ii) extracting those cells with 0.4 M TCA for chromatographic analysis. After neutralization, the TCA extracts were subjected to thin layer chromatography on PEI-cellulose sheets to identify metabolites of [^{3}H]uridine. In Fig. 10, histograms depict the distribution on chromatograms of uridine-derived ^{3}H-activity between uridine and uridine metabolites in hepatocyte extracts after graded intervals of incubation with [^{3}H]uridine at 22 °C. The data clearly show that little metabolism of uridine took place in the hepatocytes during 10 min of incubation. The results of the TLC analyses are summarized in Table 5. About 80% of the radioactivity in the extract was associated with uridine. There was no detectable radioactivity in uracil and less than 10% of the ^{3}H-activity accompanied uridine phosphates. Thus, free uridine pools were present in the hepatocytes and minor amounts of the nucleoside were incorporated into nucleotides. About 5% of the intracellular radioactivity was associated with another metabolite, perhaps dihydrouracil, but no attempt was made to identify this compound.

F. Kinetics of uridine transport by hepatocytes

The relationship of inward uridine fluxes to uridine concentration was explored in the experiment of Fig. 11. Uridine fluxes were measured as initial rates from short time courses of uridine uptake by the hepatocytes. Uridine fluxes obtained in this way are plotted against uridine concentration in Fig. 11. The Hanes-Woolf plot of these data shown in Fig. 12 is curved, suggesting that more than one process contributed to uridine fluxes. Kinetic constants were determined by non-linear regression analysis of these data (Fig. 12), using a model-fitting computer program (McPherson, 1985).

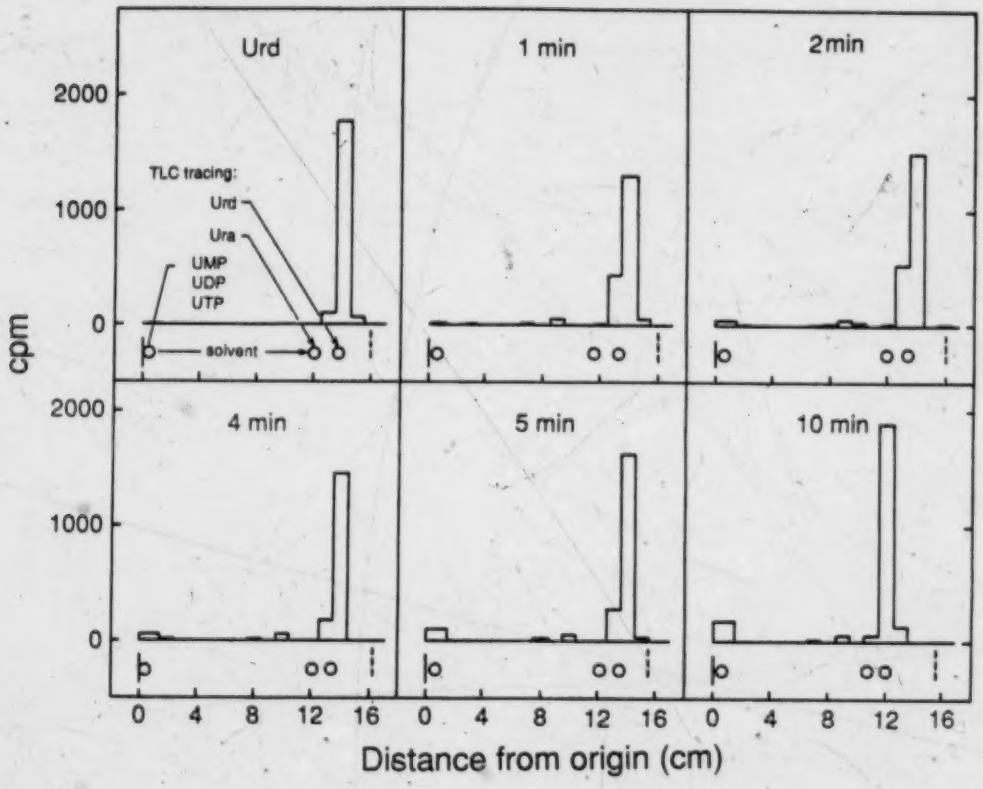


Fig. 10: Metabolism of [^{3}H]uridine in hepatocytes. The histograms depict the chromatographic distribution of ^{3}H-activity in TCA extracts of hepatocytes following incubation with [^{3}H]uridine. Incubation mixtures, which contained 4 x 10^5 hepatocytes in 400 µl of Na^+ medium with 50 µM [^{3}H]uridine, were assembled in microcentrifuge tubes over oil layers that had been placed over 100 µl of 0.4 M TCA in the tube tips. After incubation at 22 °C for the intervals specified, cells were pelleted into TCA layers. TCA extracts were neutralized and chromatographed on PEI-cellulose thin layers together with 10 nmol portions of the marker substances indicated; 0.5 cm sections of chromatogram lanes were assayed for ^{3}H-activity. Data from a typical set of chromatograms are shown. Broken lines indicate solvent fronts.

Table 6. Metabolism of [^{3}H]uridine in hepatocytes. This table summarizes chromatographic data on the formation of uridine metabolites in hepatocytes in experiments such as that described in Fig. 10; data from Fig. 10 are included in this table. A portion of the uridine content of the TCA extracts was derived from suspending medium that accompanied cells during pelleting into the TCA layer. That contribution was estimated from the [^{3}H]PEG content of TCA extracts in experiments in which incubation mixtures contained [^{3}H]PEG in place of [^{3}H]uridine. The data here reported were corrected for the uridine content of medium that accompanied hepatocytes during pelleting into oil layer. Values reported are means (± S.D.) from three experiments.

		Intracellular radioactivity (% total)		
Time (min)	TLC recovery (% applied cpm)	Urd	Phosphates	Other metabolites
0.5	103 ± 9	90 ± 3	1 ± 0.2	6 ± 2
1	96 ± 2	89 ± 3	2 ± 0.5	5 ± 2
2	97 ± 3	88 ± 3	2 ± 0.3	5 ± 2
3	86 ± 11	89 ± 1	3 ± 0.8	5 ± 2
4	90 ± 1	86 ± 3	4 ± 0.1	4 ± 0.3
5	89 ± 4	85 ± 4	6 ± 0.5	4 ± 0.1
10	89 ± 2	83 ± 2	7 ± 1	4 ± 0.1

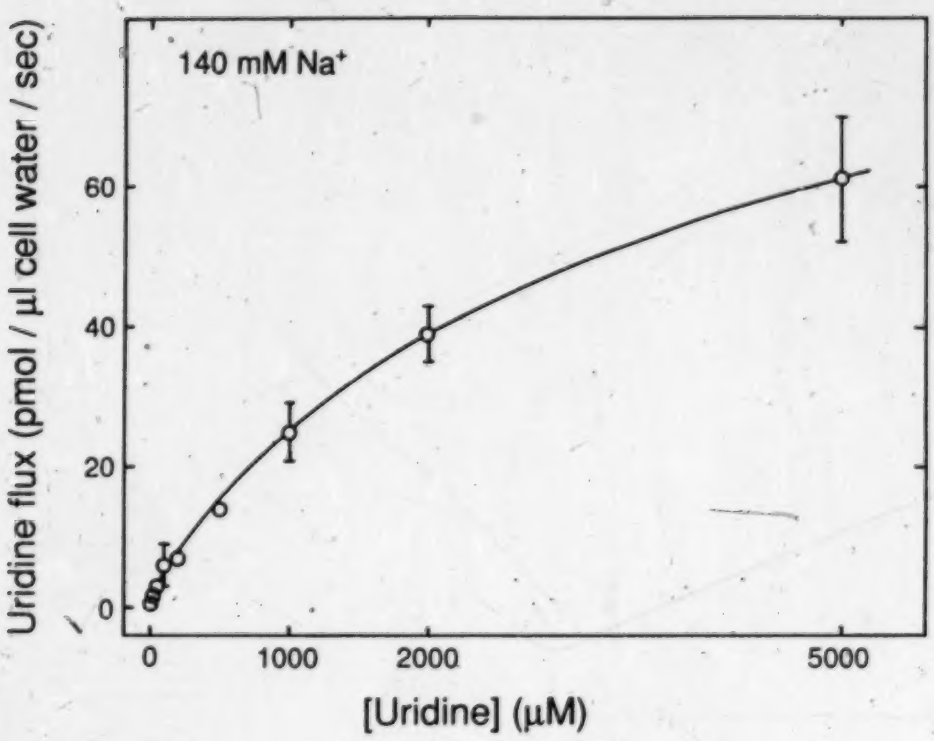


Fig. 11: Relationship between uridine concentration and rates of inward transport of uridine by hepatocytes. Using sets of replicate incubation mixtures (which, when completed, contained 2 x 10^5 hepatocytes in 200 µl of Na^+ medium with [^{3}H]uridine at one of the indicated concentrations), uridine fluxes were measured by the initial uptake rate procedure described in the text (Sec. F) and in Materials and Methods. Initial rates (i.e. uridine fluxes) were determined by fitting parabolas to time course data for uridine uptake at each of the uridine concentrations indicated. Time course assays were repeated 3 times; means (± S.D.) of the three extracted rates are plotted.

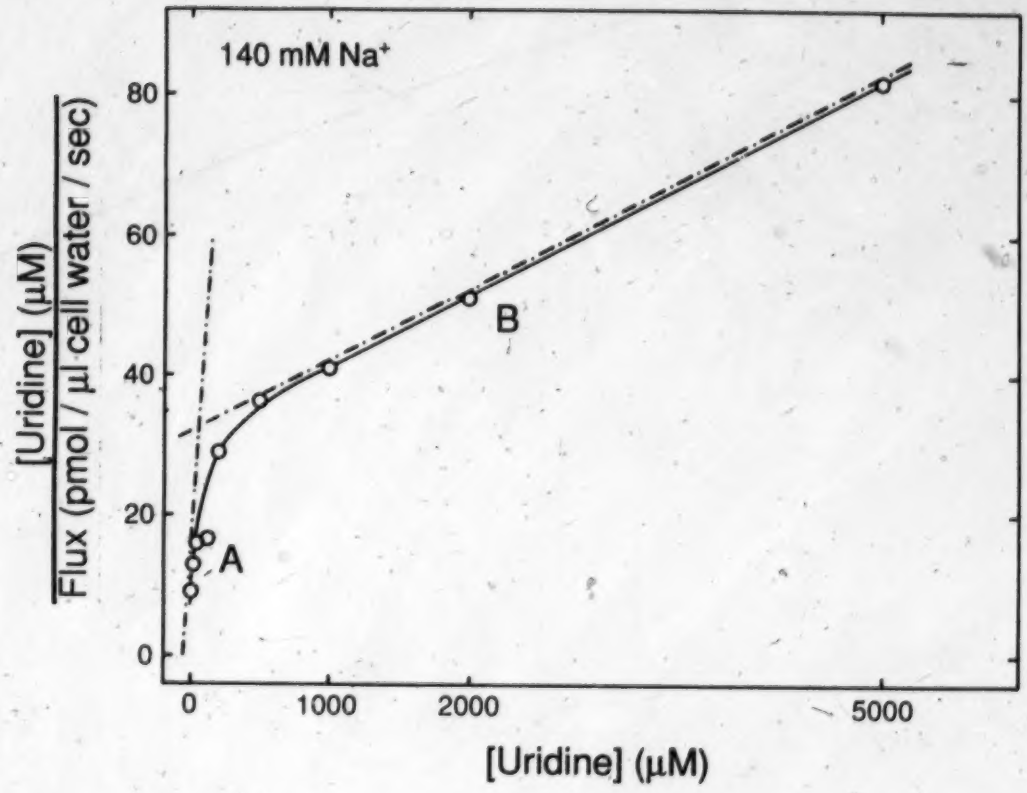


Fig. 12: Hanes-Woolf plot of the data of Fig. 11. Two processes (A, B) appear to contribute to the observed fluxes. The apparent K_m for process A is 54 μM and V_{max} is 3 pmol/μl cell water/sec; process B has an apparent K_m of 2871 μM and a V_{max} of 95 pmol/μl cell water/sec.

The apparent K_m for process A is 54 μM and the V_{max} is 3 pmol/μl cell water/sec; process B has an apparent K_m of 2871 μM and a V_{max} of 95 pmol/μl cell water/sec.

G. Inhibition of thymidine transport by NBMPR

A tactic used to study nucleoside fluxes independently of metabolism is that of employing non-metabolized permeant in uptake studies (Jakobs and Paterson, 1986; Paterson and Cass, 1986). Thymidine was employed as a permeant in the present study because that nucleoside is poorly metabolized in hepatocytes (Ungemach and Hegner, 1978; Holstege *et al.*, 1985). The availability of non-metabolized permeants also facilitates demonstration of concentrative uptake processes (Jakobs and Paterson, 1986; Dagnino *et al.*, 1987; Vijayalakshmi and Belt, 1988).

The effect of NBMPR on thymidine uptake by hepatocytes in Na^+ medium was explored in the experiment of Fig. 13. The cellular uptake of [^{3}H]thymidine during short time courses (upper panel) and long time courses (lower panel) was inhibited by about 50% in the presence of 10 μM NBMPR. After 5 min of incubation in the absence of inhibitor, intracellular concentrations of thymidine equivalents were about twice that of the medium; however, when the medium contained NBMPR, the intracellular levels of thymidine were about the same as in the external medium. Fig. 14 shows that NBMPR had only minor inhibitory effects on thymidine uptake by hepatocytes in K^+ medium during short intervals (0 to 30 sec) of incubation at 22 °C, but when time courses were extended to 5 min, reductions in cellular levels of thymidine of about 30% were evident.

To further discriminate between Na^+-linked and Na^+-independent NT activity, uptake studies were conducted in medium in which NMG^+ replaced Na^+. As shown in Figs. 15 and 16, levels of thymidine achieved in 5 min by hepatocytes in NMG^+ medium were several-fold higher than in Na^+ and K^+ media. Expressed as a fraction of the final values achieved, the inhibitory effect of 10 μM NBMPR on thymidine uptake by hepatocytes in NMG^+ medium was substantially less than that in Na^+ medium, as is evident in comparison of Figs. 13 and 15. However, in molar terms, the changes in cellular thymidine content that

resulted from the presence of NBMPR during 30 sec and 5 min of exposure to [^{3}H]thymidine were about the same in Na^+ and NMG^+ media. Thymidine fluxes in NMG^+ medium were about 1.5 times those in Na^+ medium. This remarkable enhancement of thymidine fluxes in hepatocytes in NMG^+ medium was confirmed in several similar experiments, but has not been explored further with other nucleoside permeants.

Other studies in this laboratory have shown that NMG^+ media did not support Na^+-linked transport of purine nucleosides in two lines of cultured cells, IEC-6 intestinal epithelial cells (Jakobs and Paterson, 1986) and mouse leukemia L1210 cells (Dagnino *et al.*, 1987). Accordingly, the NMG^+ stimulation of thymidine fluxes was unexpected and requires further investigation. It is possible that the NMG^+ cation may serve in place of Na^+ as a co-substrate in a concentrative thymidine transport process. It may be noted that Vijayalakshmi and Belt (1988) have described two Na^+-linked, concentrative nucleoside transport processes in mouse enterocytes which differ in their specificity for thymidine.

Fig. 16 compares time courses for the uptake of thymidine by hepatocytes in Na^+, K^+ and NMG^+ media; these data are replotted from Figs. 13-15. NMG^+ enhancement of thymidine fluxes is evident in these data. While the lower uptake rates in K^+ medium would argue for Na^+-dependency, the results in NMG^+ medium indicated otherwise. Table 7 summarizes the results of thymidine flux measurements conducted in the presence and absence of 10 μM NBMPR in 10 experiments conducted under the conditions of Figs. 13-15. In Na^+ medium, thymidine fluxes were reduced by about 40% in the presence of NBMPR. Fluxes were not changed significantly in K^+ medium by the presence of NBMPR. In NMG^+ medium, thymidine fluxes were higher than in either Na^+ medium or K^+ medium, and NBMPR reduced this rate by about 23%. Thus, the NBMPR-sensitive component of the thymidine transport machinery appears to be distinct from that component influenced by replacing Na^+ with K^+ or NMG^+. It is also possible that low thymidine fluxes in K^+ medium masked NBMPR effects.

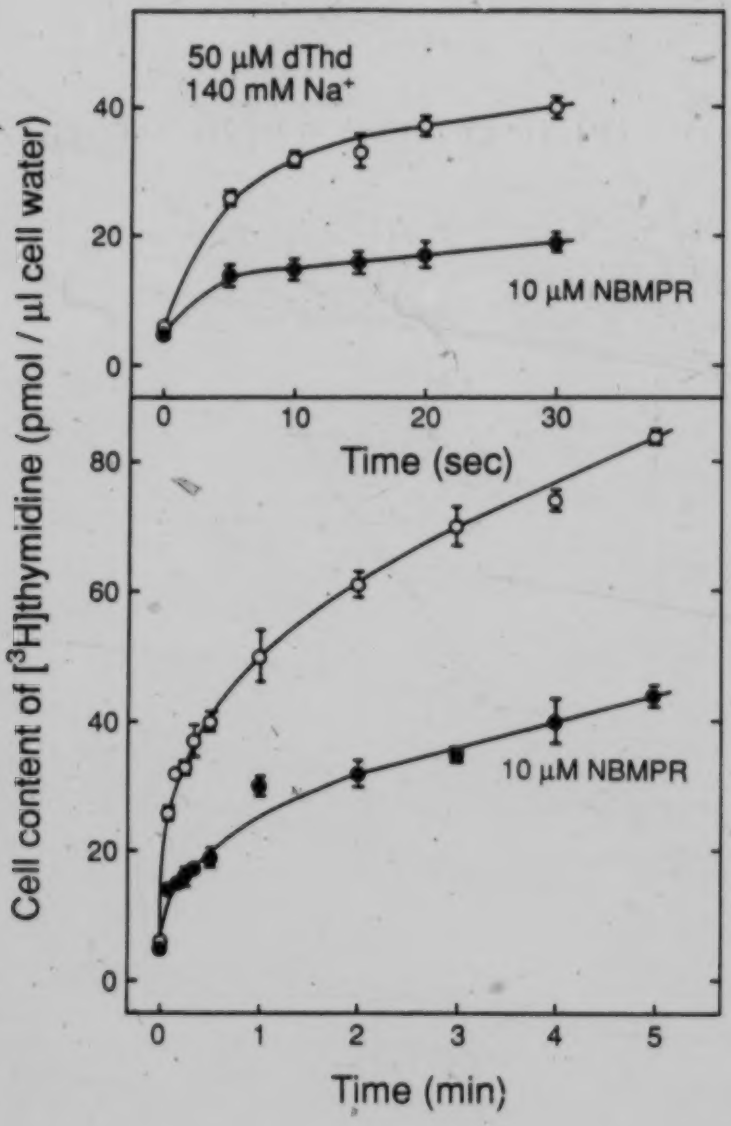


Fig. 13: NBMPR inhibition of thymidine uptake by hepatocytes in Na^+ medium. Replicate suspensions of freshly-isolated cells were incubated for the time intervals specified in medium containing 140 mM Na^+ and 50 μM [^{3}H]thymidine with (●) and without (○) 10 μM NBMPR. Cell-associated ^{3}H was determined as described in Materials and Methods. The data are means (± S.D.) of triplicate determinations. These results are typical of 3 other similar experiments.

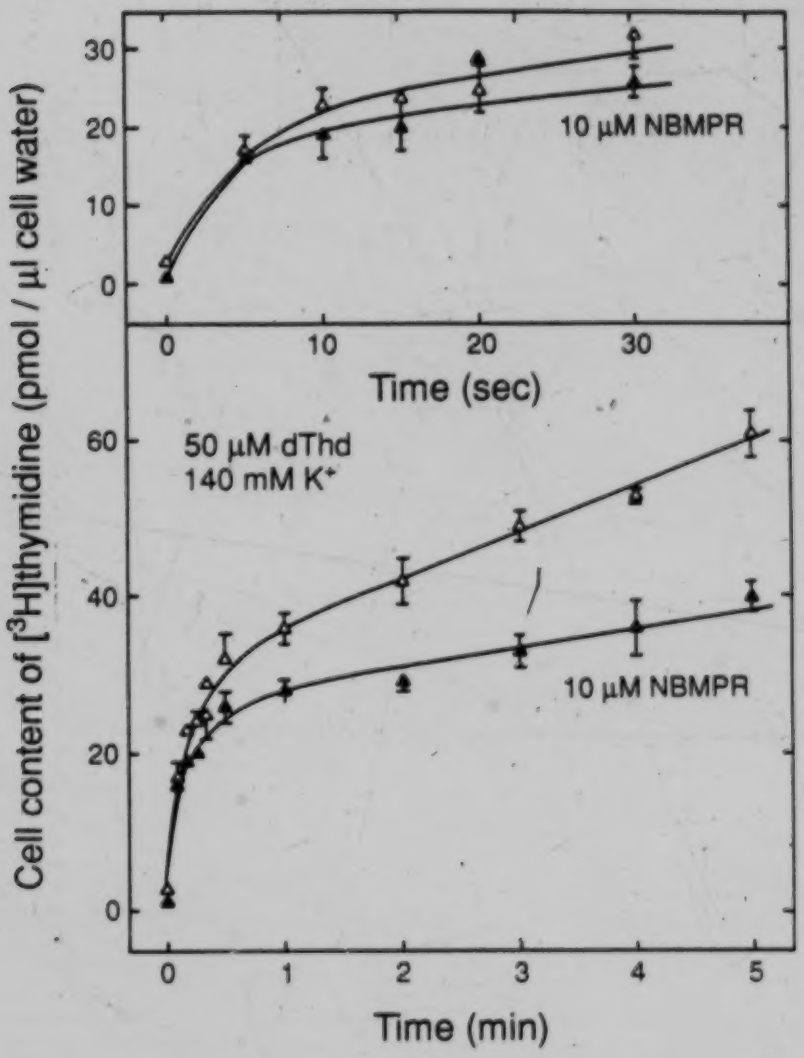


Fig. 14: NBMPR inhibition of thymidine uptake by hepatocytes in K^+ medium. Replicate suspensions of freshly-isolated hepatocytes were incubated for the time intervals specified in medium containing 140 mM K^+ and 50 µM [^{3}H]thymidine with (▲) and without (△) 10 µM NBMPR. Cell-associated ^{3}H was determined as described in Materials and Methods. The data are means (± S.D.) of triplicate determinations. These results are typical of 2 other similar experiments.

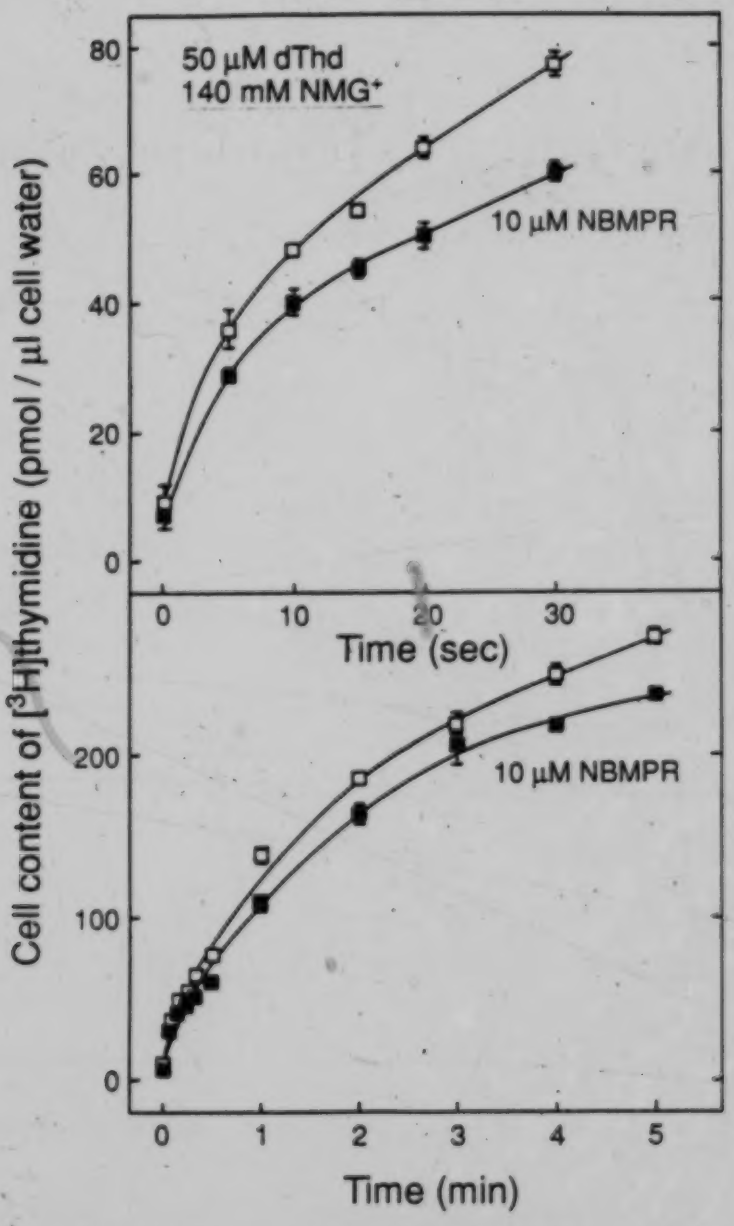


Fig. 15: NBMPR inhibition of thymidine uptake by hepatocytes in NMG^+ medium. Replicate suspensions of freshly-isolated hepatocytes were incubated for the time intervals specified in medium containing 140 mM NMG^+ and 50 µM [^{3}H]thymidine with (■) and without (□) 10 µM NBMPR. Cell-associated ^{3}H was determined as described in Materials and Methods. The data are means (± S.D.) of triplicate determinations. These results are typical of 2 other similar experiments.

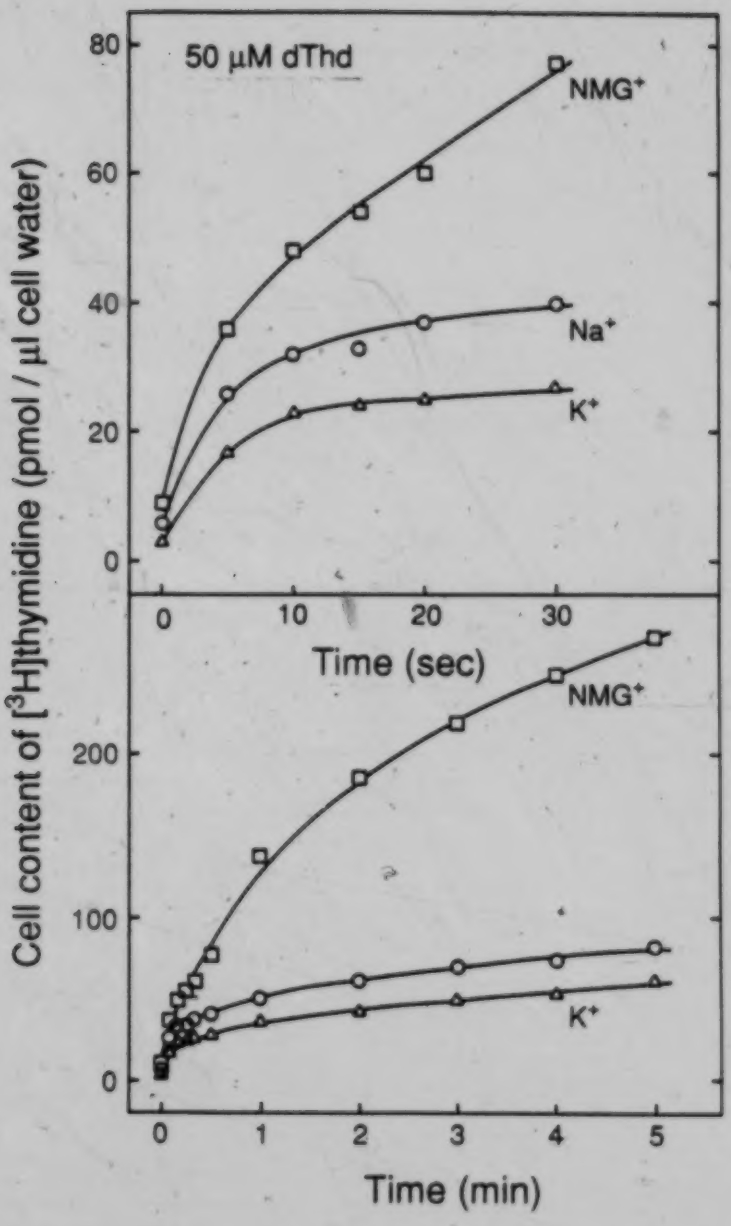


Fig. 16: Cation-dependent uptake of thymidine by hepatocytes. Time courses of [^{3}H]thymidine (50 μM) uptake by hepatocytes suspended in Na^+ (○), K^+ (△), or NMG^+ (□) medium. These data are replotted from Figs. 13-15.

Table 7. Influence of NBMPR on inward thymidine (50 μM) fluxes in hepatocytes. The data shown include results from the experiments of Figs. 13-15. Mean fluxes (± S.E.M.) are listed; when Na^+ medium was used, n = 4, otherwise n = 3.

	Thymidine fluxes (pmol/μl cell water/sec)		
Transport medium	With NBMPR (10 μM)	Without NBMPR	Student's *t*-test
Na^+	1.7 ± 0.3	2.7 ± 0.2	($p < 0.05$)
K^+	1.7 ± 0.5	2.2 ± 0.4	(N.S.)
NMG^+	2.8 ± 0.2	3.6 ± 0.2	($p < 0.05$)

H. Relationship between NBMPR concentration and inhibitory effects on thymidine transport

In exploring effects of graded NBMPR concentrations on thymidine fluxes in hepatocytes, cells were equilibrated with designated NBMPR concentrations for 10 min at 22 °C prior to flux assays. Fluxes, defined as initial rates of permeant uptake (Paterson and Cass, 1986), were estimated from measurements of [^{3}H]thymidine uptake by hepatocytes during graded intervals (0-7.5 sec) of exposure to 20 μM [^{3}H]thymidine. Initial rates of thymidine uptake were determined from the coefficients of the first order term in parabolas fitted to the uptake data by polynomial regression analysis. This procedure is equivalent to measuring the time-zero slope of the time course of the cellular uptake of thymidine. In Fig. 17, thymidine fluxes, expressed as percentages of control fluxes (measured in cells without NBMPR treatment) are plotted against NBMPR concentration (without correction for cellular uptake of NBMPR). A biphasic relationship is apparent in these data. At NBMPR concentrations below 10 nM, thymidine fluxes were sensitive to inhibition by NBMPR with an IC_{50} of about 0.2 nM. At NBMPR concentrations between 10 nM and 1 μM, the inhibition of thymidine transport remained constant at about 35% of control rates; however, NBMPR levels above 10 μM caused further reduction of thymidine fluxes. These results indicate that a component of thymidine permeation was of high NBMPR sensitivity.

I. Inhibition of thymidine transport by uridine

Figs. 8, 9 and 13 indicate that rates of uridine and thymidine transport by hepatocytes in Na^+ medium were generally similar, and that both nucleosides accumulated in the cells to levels about two-fold higher than external concentrations.

To determine if transporters that mediate thymidine entry in the hepatocytes might also mediate uridine entry, thymidine fluxes were measured in the presence of graded uridine concentrations. The data of Table 8 demonstrate that uridine inhibited thymidine fluxes in hepatocytes with an IC_{50} of about 100 μM. At a uridine concentration of 250 μM, thymidine fluxes were reduced to about 45% of control values, and to less than 8% by 2 mM uridine. It is concluded that the thymidine transporter accepts uridine as a substrate.

J. A "*trans* effect" in thymidine transport

Trans effects refer to the influence of a permeant at the *trans*[4] face of a membrane on fluxes of that permeant (or related permeant) in the *cis* to *trans* direction, that is, from the opposite membrane face. *Trans* effects have been reported for the equilibrative transporters that mediate glucose and nucleoside transport in human erythrocytes (Baker and Widdas, 1973; Wohlhueter and Plagemann, 1982; Plagemann *et al.*, 1982; Jarvis *et al.*, 1983; Jarvis, 1986b). Examples of *trans* phenomena in erythrocytic NT are *trans* acceleration, countertransport, and the achievement of maximum velocities in equilibrium exchange diffusion fluxes that are greater than zero-*trans* fluxes. To demonstrate a *trans* effect in hepatocytes, inward fluxes of thymidine were compared in hepatocytes that were either "loaded" with non-isotopic thymidine or not loaded. As is indicated in Fig. 18, when rates of uptake of 10 μM [^{3}H]thymidine was measured in hepatocytes loaded with 1 mM non-isotopic substrate, levels of cellular [^{3}H]thymidine increased more rapidly than in comparable experiments with cells that were not thymidine loaded. Thus, the presence of thymidine on the *trans* side of the membrane accelerated the influx of [^{3}H]thymidine: inward fluxes of [^{3}H]thymidine (0.12 pmol/μl cell water/sec in unloaded cells) were doubled (0.28 pmol/μl cell water/sec) in cells loaded with 1 mM thymidine.

K. The metabolism of thymidine in hepatocytes

Levels of thymidine kinase activity in liver are low in adult rats relative to those in neonatal liver and in other proliferating cells (Ferdinandus *et al.*, 1971; Kit, 1976); however, levels of thymidine phosphorylase activity do not follow this pattern. Ungemach and Hegner (1978) reported that when rat hepatocytes were incubated with low (5 μM) and high (1 mM) concentrations of [^{3}H]thymidine at 37 °C for 2 hrs, little thymine was formed. To the contrary, Holstege *et al.*, (1985) did not detect conversion of thymidine to thymine by

[4] In this convention, cross-membrane fluxes of permeant are understrood to proceed in the *cis* to *trans* direction and *cis* may refer to the solution at either membrane face. Zero-*trans* influx refers to the condition in which permeant concentration at a designated membrane face, the external membrane face in this example, is initially zero, while that at the other face is varied.

hepatocytes in suspension. To detect thymidine cleavage in the present study, hepatocytes were incubated with 50 μM [^{3}H]thymidine for specified time periods and neutralized TCA extracts of cells were analyzed by thin-layer chromatography on PEI-cellulose sheets. The data of Fig. 19 show that formation of [^{3}H]thymine from [^{3}H]thymidine in the hepatocytes was a minor process that progressed with time. The results of the TLC analyses are summarized in Table 9. After 10 min of incubation, about 80% of the [^{3}H]thymidine content of the hepatocytes was in the form of free (unmetabolized) thymidine. About 90% of the radioactivity applied to the chromatograms accompanied the marker compounds.

L. Kinetics of thymidine transport in hepatocytes

In exploring relationships between the extracellular concentration of thymidine and inward fluxes of that nucleoside in hepatocytes, fluxes were measured as initial rates of cellular uptake of [^{3}H]thymidine, as in the experiment of Fig. 17. Thymidine fluxes measured in this way are plotted against concentration in Fig. 20. A plot of these data in the form of the Hanes-Woolf transformation of the Michaelis-Menten equation is presented in Fig. 21. The curved plot indicates that more than one permeation process contributed to the observed thymidine fluxes. Kinetic constants were determined by a model-fitting computer program (McPherson, 1985). The apparent K_m for process A (high-affinity process) is 30 ± 16 μM (S.E.M) and the V_{max} is 0.4 ± 0.2 pmol/μl cell water/sec; process B (low-affinity process) has a K_m of 2572 ± 587 μM and a V_{max} of 115 ± 14 pmol/μl cell water/sec. The K_m values differ from those reported by Ungemach and Hegner (1978) [high-affinity system: K_m, 5.3 μM; V_{max}, 0.47 pmol/10^6 cells/sec, and low-affinity system: 480 μM, 37.6 pmol/10^6 cells/sec respectively], however, the present data confirm the earlier finding of low- and high-affinity systems for thymidine transport in rat hepatocytes.

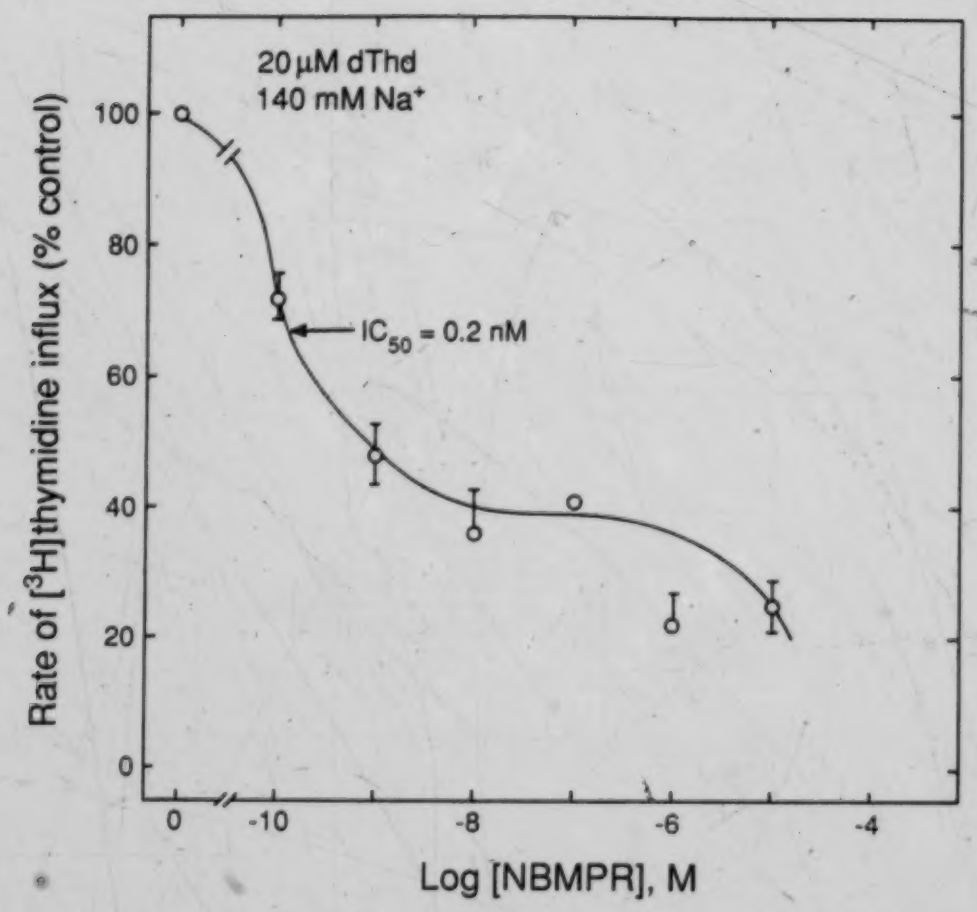


Fig. 17: NBMPR inhibition of thymidine fluxes in hepatocytes. Replicate assay mixtures containing 2 x 10^5 cells in 100 μl Na^+ medium with and without NBMPR (control) at the indicated concentrations were incubated for 10 min at 22 °C before addition of 100 μl of medium containing [^{3}H]thymidine (20 μM, final) to initiate intervals of thymidine uptake. Inward fluxes of [^{3}H]thymidine were assayed as described in Materials and Methods. The control rate of thymidine influx was 3.2 pmol/μl cell water/sec. The data are means (± S.D.) of triplicate determinations.

Table 8. Uridine inhibition of thymidine transport in hepatocytes. Completed assay mixtures contained 2 x 10^5 cells in 200 μl Na^+ medium with 5 μM [^{3}H]thymidine and were prepared with or without uridine at the indicated concentrations. Replicate assay mixtures in sets, each with a particular uridine concentration, were employed to determine time courses of [^{3}H]thymidine uptake, as in Fig. 9. Time courses were analyzed as in Fig. 11 to measure initial rates of thymidine uptake (that is, thymidine fluxes) which are here expressed as percentages of the thymidine fluxes (0.3 pmol/μl cell water/sec) in control assay mixtures (without uridine). The data are means (± S.D.) of triplicate assays.

Uridine, μM	Thymidine flux (% of flux in the absence of Urd)
10	73 ± 2.1
50	70 ± 0.7
100	55 ± 3.5
250	45 ± 8.5
1000	16 ± 4.9
2000	8 ± 3.5

IC_{50} = 100 μM

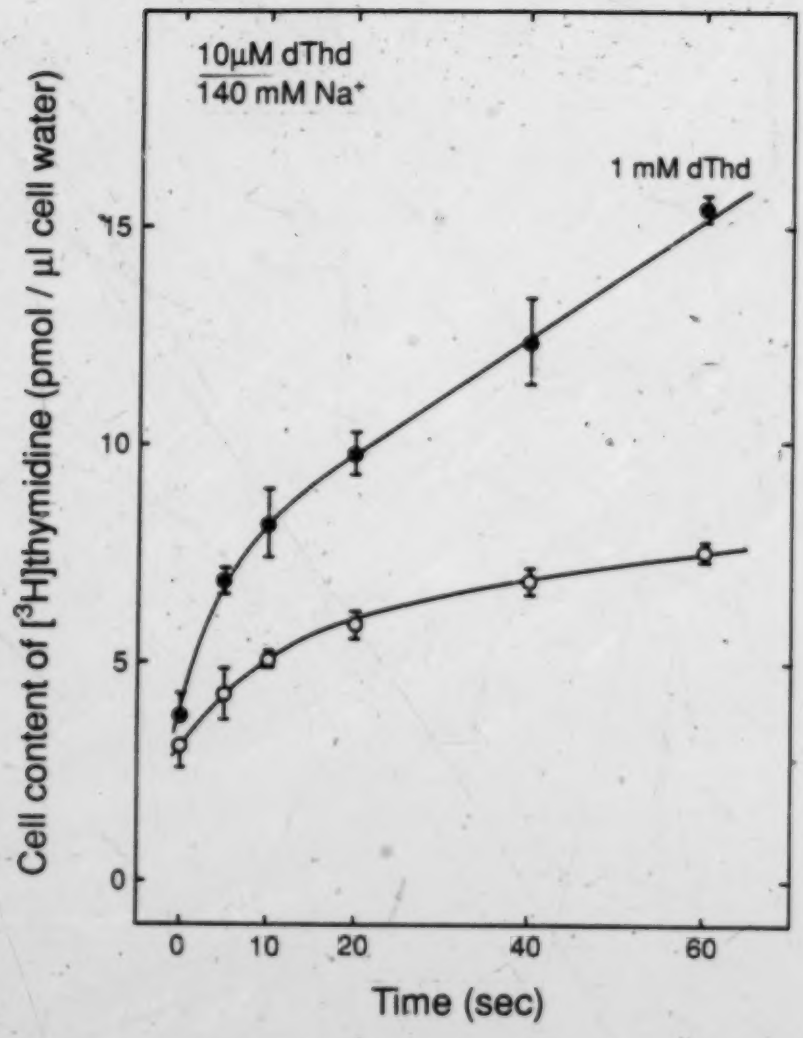


Fig. 18: "Counter-transport" of [^{3}H]thymidine: a *trans* effect in the membrane transport of [^{3}H]thymidine by rat hepatocytes. Replicate assay mixtures containing 2 x 10^5 cells in 100 μl of Na^+ medium with (●) or without (○) 1 mM thymidine were incubated for 10 min at 22 °C. Intervals of thymidine uptake were begun by adding 100 μl of 20 μM [^{3}H]thymidine in Na^+ medium and ended by pelleting cells under oil, as in the experiment of Fig. 13. The data are means (± S.D.) of triplicate assays.

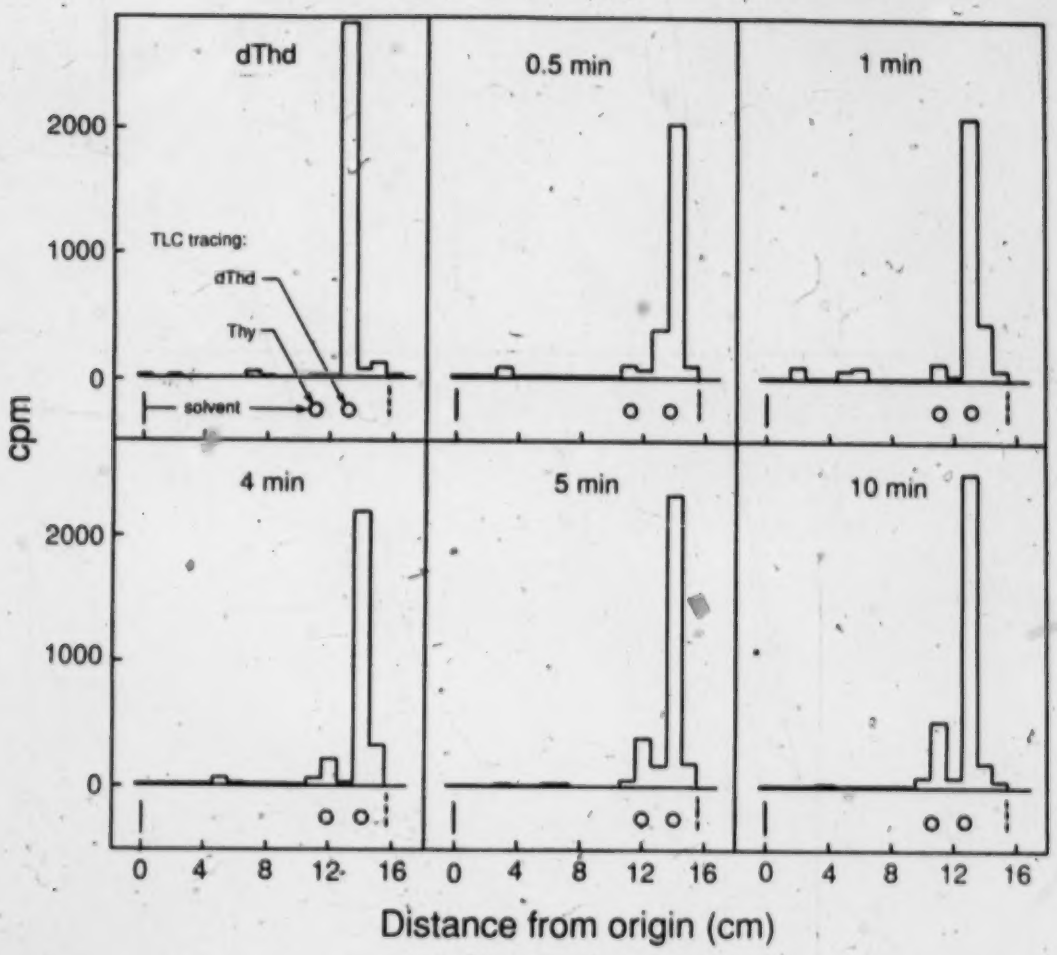


Fig. 19. Metabolism of [^{3}H]thymidine in hepatocytes. In an experiment similar to that of Fig. 10, hepatocytes were incubated at 22 °C with 50 μM [^{3}H]thymidine in Na^+ medium for the periods indicated, which were ended by pelleting cells through an oil layer into TCA. Neutralized TCA extracts were chromatographed on thin layers of PEI-cellulose and 0.5 cm sections of chromatogram lanes were assayed for ^{3}H-activity. Tracings of typical chromatograms are shown together with histograms depicting their distribution of ^{3}H-activity. Broken lines indicate solvent fronts.

Table 9: Metabolism of [^{3}H]thymidine in hepatocytes. This table summarizes chromatographic data on the formation of thymidine metabolites in hepatocytes in experiments similar to that described in Fig. 19. Data from Fig. 19 are included in this table. A portion of the thymidine content of the TCA extracts was derived from suspending medium that accompanied cells during pelleting into the TCA layer. This contribution was estimated from the [^{3}H]PEG content of TCA extracts in experiments in which incubation mixtures contained [^{3}H]PEG in place of [^{3}H]thymidine. Values reported are means (± S.D.) from three experiments.

Time (min)	TLC recovery (% of applied cpm)	Intracellular radioactivity (% total)	
		Thymine	Thymidine
0.5	93 ± 2	5 ± 1	95 ± 1
1	91 ± 1	5 ± 2	95 ± 2
2	92 ± 6	4 ± 0.4	93 ± 5
3	93 ± 1	6 ± 1	92 ± 2
4	89 ± 2	10 ± 3	90 ± 3
5	90 ± 6	13 ± 4	87 ± 4
10	90 ± 3	19 ± 2	81 ± 2

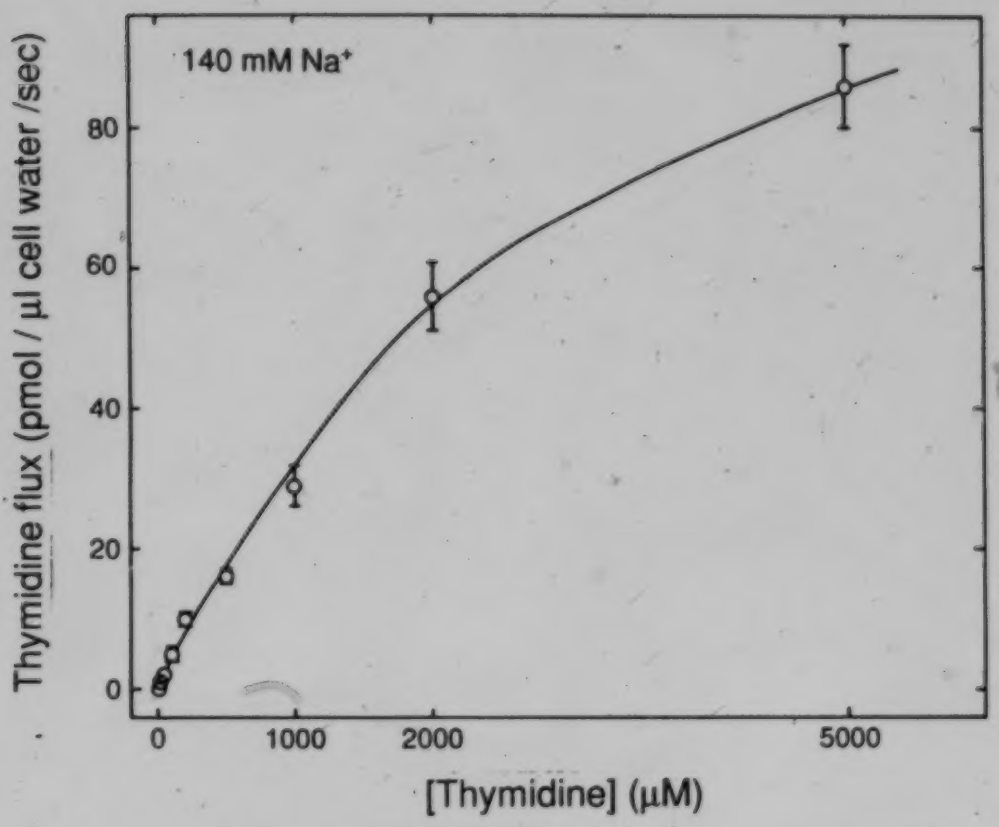


Fig. 20: Relationship between thymidine concentration and inward fluxes of thymidine in hepatocytes. In an experiment similar to that of Fig. 11, thymidine fluxes were measured at 22 °C in hepatocytes suspended in Na^+ medium containing [^{3}H]thymidine at the concentrations indicated. Time course assays were repeated 3 times; means (± S.D.) of the three extracted rates are plotted.

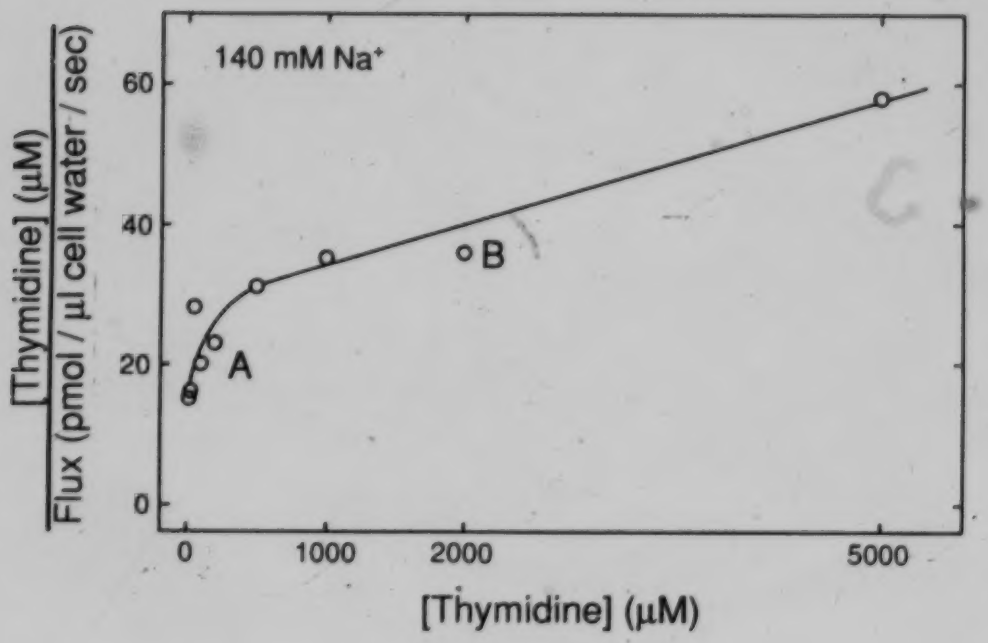


Fig. 21: Hanes-Woolf plot of data of Fig. 20. Two processes (A, B) appear to contribute to the observed fluxes. The apparent K_m for process A is 30 ± 16 µM and the V_{max} 0.4 ± 0.2 pmol/µl cell water/sec; process B has a K_m of 2572 ± 587 µM and a V_{max} of 115 ± 14 pmol/µl cell water/sec.

V. DISCUSSION

The original procedures for the preparation of hepatocytes using the two-step collagenase perfusion method yielded suspensions that contained susbstantial quantities of damaged cells and cell debris, together with a small fraction of intact cells that were "viable" by the criteria of dye exclusion and refractility under microscopic examination (Berry and Friend, 1969; Seglen, 1973; Williams *et al.*, 1977). The introduction of the Percoll gradient procedure enabled isolation of metabolically active hepatocytes with intact membranes, essentially free of damaged cells and cell debris (Pertoft and Laurent, 1977; Dalet *et al.*, 1982; Kreamer *et al.*, 1986). In using the Percoll procedure in the present study, yields of "viable" cells were comparable to those reported by other workers using this method (Kreamer *et al.*, 1986). The isolation procedure yielded homogeneous preparations of hepatocytes and therefore, precluded interference from other types of liver cells.

A. High-affinity NBMPR binding in hepatocytes

NBMPR, a very useful probe of the equilibrative NT mechanism of erythrocytes and the best studied of the 6-thiopurine NT inhibitors, binds tightly to specific cellular sites, which are evidently located on NT elements of the plasma membrane (Pickard *et al.*, 1973; Cass *et al.*, 1974; Jarvis and Young, 1980; Gati and Paterson, 1989). In human erythrocytes, the occupancy of these sites by NBMPR is directly related to inhibition of NT (Cass *et al.*, 1974; Jarvis *et al.*, 1982).

In the Jarvis-Young model of the erythrocyte NT system, sites for nucleoside permeation and for high-affinity binding of NBMPR are visualized as overlapping domains on the NT protein, a band 4.5 glycoprotein with a relative molecular mass of 45,000 - 65,000 (Jarvis and Young, 1981; Young *et al.*, 1983 and 1984). While some features of that model may be challenged, it is a reasonable basis for starting molecular studies with erythrocyte NT systems. However, NT systems in nucleated cells are proving to be complex and the Jarvis-Young model is not able to accommodate a number of observations with nucleated cells. For example, (i) cultured Walker 256 carcinosarcoma cells are devoid of NBMPR

binding sites, yet exhibit equilibrative NT activity (Belt and Noel, 1985), (ii) NBMPR effects on NT activity in lymphoma S49 cells may be modified genetically without change in NT activity (Cohen *et al.*, 1985; Aronow *et al.*, 1985), and (iii) in several lines of cultured hepatoma cells, NBMPR occupancy of high affinity binding sites is without substantial effect on NT activity (Paterson *et al.*, 1987). Considerable diversity in NT mechanisms has been recognized in nucleated cells and the "erythrocytic type" of NT activity (equilibrative, high NBMPR sensitivity) is but one of several transport systems that mediate nucleoside permeation in these cells. The association of (i) binding sites with high affinity for NBMPR and (ii) NT activity of high NBMPR sensitivity has been clearly shown in S49 cells, by Cohen *et al.* (1985) who reported the loss of both properties in the single-step mutant clone, AE_1. NT processes of high NBMPR sensitivity have been shown in various other cell lines that also express NBMPR binding sites. Thus, it is a reasonable first assumption that sites with high affinity for NBMPR are associated with equilibrative nucleoside transporters. However, it is necessary to relate the presence of binding sites to NT activity in each cell type studied, since no generalizations can be made. NBMPR binding sites are widely distributed in cells and tissues.

The data presented in Figs. 4 and 5 indicate the presence of high-affinity NBMPR binding sites on Percoll-isolated hepatocytes. The binding constants, K_D and B_{max}, here reported compare reasonably with those reported earlier by Plagemann and Wohlhueter (1985) for Percoll-isolated rat hepatocytes. Numbers of binding sites per cell differed, but were of the same order (about 10^5 sites/cell). The presence on hepatocytes of sites that bind NBMPR suggests the presence of NBMPR-sensitive transporters. It may be noted that the joint presence in several lines of hepatoma cells of (i) NT systems of low NBMPR sensitivity ($IC_{50} > 1\ \mu M$), and (ii) sites that bind NBMPR with high affinity, has given rise to the concept of "uncoupled" NBMPR binding sites (Ng, 1986; Paterson *et al.*, 1987). The present study appears to have revealed coupling of NBMPR binding sites to Na^+-linked NT mechanisms in rat hepatocytes.

B. Nucleoside permeation in hepatocytes

The enzymatic and transport properties of the several membrane domains that exist *in situ* are probably expressed in suspensions of isolated hepatocytes. Thus, if the sinusoidal, lateral and bile duct canalicular regions of the hepatocyte plasma membrane have specialized NT activities, those activities would likely be expressed in hepatocyte suspensions, whereas, in the intact tissue, those activities would probably be parts of reaction sequences involved in the hepatic processing of nucleoside/nucleobase constituents of plasma. It is possible that the NT machinery of these specialized membrane domains may differ fundamentally, as is the distribution in renal epithelial cells of Na^+-dependent and equilibrative glucose transporters betweeen the apical and basolateral regions of the plasma membrane (Baker and Widdas, 1973; Misfeldt and Sanders, 1985).

Because several types of NT mechanisms have so far been recognized (equilibrative transporters of high or low sensitivity to NBMPR, and Na^+-linked, concentrative nucleoside transporters) one may reasonably expect to find one or more of these activities expressed in freshly isolated hepatocytes. In general, the substrate specifities of equilibrative NT systems explored hitherto have been broad. Na^+-linked NT systems have been recognized in epithelial cells of several types and in lymphoid cells (splenocytes and L1210 mouse leukemia cells), but their distribution may be less widespread than that of equilibrative NT systems of high NBMPR sensitivity. Because of their comparatively recent recognition, exploration of the Na^+-linked systems has been limited and, accordingly, substrate specificities have not been extensively explored. There is some evidence that substrate specificities of Na^+-linked transporters may differ from one cell type to another. Thus, Ungemach and Hegner (1978) described the Na^+-dependent, concentrative uptake of thymidine in hepatocytes, whereas the Na^+-linked NT systems in cultured IEC-6 intestinal epithelial cells and in cultured mouse leukemia L1210 cells do not transport thymidine (Jakobs and Paterson, 1986; Dagnino *et al.*, 1987). Vijayalakshmi and Belt (1988) have reported the presence in mouse intestinal epithelial cells of two different Na^+-linked transport systems distinguishable in their relative abilities to transport formycin B and thymidine. Accordingly, one might expect complexity in the

expression of NT activity. The Na^+-linked NT systems so far reported, are listed in Table 10.

C. NBMPR sensitivity of pyrimidine nucleoside transport

The present study shows that fluxes of uridine (Figs. 6, 9) and thymidine (Fig. 13) into hepatocytes were partially inhibited by NBMPR. This finding is consistent with an earlier report from this laboratory which indicated that an NBMPR-sensitive transport system contributed to cytidine entry into perfused mouse liver (Kolassa and Paterson, 1982). As summarized in Tables 5 and 7, uridine (100 μM) and thymidine (50 μM) fluxes into hepatocytes in Na^+ medium were roughly halved in the presence of 10 μM NBMPR. The NBMPR-insensitive components of uridine and thymidine permeation are considered below.

Uridine permeation in rat hepatocytes: Inward uridine fluxes of low NBMPR sensitivity were demonstrated in the experiments of Figs. 6 and 9. As well, a component of uridine flux was Na^+-linked (Fig. 8). Na^+-linked fluxes of purine nucleosides in other cells have been found to be of low NBMPR sensitivity (Spector and Huntoon, 1984; Jakobs and Paterson, 1986; Darnowski *et al.*, 1987; Dagnino *et al.*, 1987). Non-mediated permeation of uridine could also contribute to the uridine entry component of low NBMPR sensitivity.

The uptake of 100 μM uridine by the hepatocytes was progressive and partly inhibited by 1 μM NBMPR, as seen in Fig. 6. That inhibition and the presence of high-affinity NBMPR binding sites on hepatocytes suggest the presence of an NBMPR-sensitive NT system. A concentration-effect study of the NBMPR inhibition of uridine permeation in these cells (Fig. 7) revealed the operation of permeation routes of high sensitivity (IC_{50} = 0.5 nM) and low sensitivity (IC_{50} > 10 μM) to NBMPR, suggesting that NBMPR inhibited an influx component through impairment of transporter function, possibly an equilibrative transporter.

Table 10: Na^+-dependent NT systems.

Cell type or system	Test substrates	Reference
Rat kidney (proximal tubule vesicles)	adenosine	Le Hir and Dubach, 1984
Guinea pig intestinal epithelial cells	uridine	Schwenk *et al.*, 1984
IEC-6 cells	formycin B	Jakobs and Paterson, 1986
Murine splenocytes	uridine	Darnowski *et al.*, 1987
L1210 cells	adenosine	Dagnino *et al.*, 1987
Mouse enterocytes[1]	formycin B, thymidine	Vijayalakshmi and Belt, 1988

[1] Two different sodium-linked transport mechanisms have been recognized.

These results confirm the report by Plagemann and Wohlhueter (1985) showing that in rat hepatocytes a component of uridine transport is NBMPR sensitive (IC_{50} < 10 nM) but another process contributing to uridine transport, however, was resistant to NBMPR.

Thymidine permeation in rat hepatocytes: As is evident in Fig. 13, a component of thymidine influx was inhibited by NBMPR. The concentration-effect study of the NBMPR inhibition of thymidine influx in hepatocytes shown in Fig. 17 indicates that the NBMPR-sensitive (IC_{50}, 0.2 nM) and NBMPR-insensitive (IC_{50} > 10 μM) transport mechanisms were functioning in the hepatocytes. In the present study, the occupancy by NBMPR of the high-affinity sites resulted in substantial inhibition of uridine and thymidine permeation into hepatocytes. These characteristics indicate the interaction of sites for NBMPR binding and nucleoside permeation.

A transport property of carrier-mediated processes is competitive inhibition of fluxes by structurally related substrates (Hoffer, 1977). If different nucleosides are substrates for the same carrier, the presence of one may, by competition, reduce fluxes of the other. The data of Table 8 indicate that uridine reduced [^{3}H]thymidine fluxes in hepatocytes, suggesting that both nucleosides enter cells, to some extent, by the same mechanism. Thus, uridine inhibited thymidine fluxes, probably as a competitive permeant.

The experiment of Fig. 18 showed that intracellular thymidine enhanced the influx of [^{3}H]thymidine. In this "*trans* effect" experiment, cells were incubated with 1 mM non-isotopic thymidine in order to achieve substantial intracellular levels of free thymidine before measuring inward fluxes of [^{3}H]thymidine, which were enhanced in the thymidine-loaded cells. The interpretation of experiments of this type conducted in cells with equilibrative NT (erythrocytes) systems is based on the hypothesis that transporter elements of the "simple" carrier re-orients more rapidly in the permeant-loaded state than in the unloaded state. Thus, permeant fluxes (i) in the inward direction may be affected by the intracellular concentration of that permeant, and (ii) in the outward direction by the extracellular concentration of the permeant, if transport in both directions is mediated by the same carrier species. The loaded/unloaded asymmetry is the basis of the *trans* effects. *Trans*

effects have been used to identify substrates for transport systems (Cass and Paterson, 1972 and 1973; Plagemann *et al.*, 1982) and to show that permeant fluxes are transporter-mediated.

In the present study, inward fluxes of [^{3}H]thymidine in hepatocytes were more rapid if the hepatocytes contained thymidine (Fig. 18). This experiment demonstrated net movement of [^{3}H]thymidine molecules into cells against a concentration gradient, hence the term "counterflow". In the unloaded cells, the re-orientation of the unloaded carrier appeared to be slower than in thymidine-loaded cells, hence the overall rate of [^{3}H]thymidine transport would be determined by the mobility of the unloaded carrier. In thymidine-loaded cells, however, the carrier supposedly moved in both directions in its loaded form so that the rate of transport of [^{3}H]thymidine would be determined by the mobility of the carrier-substrate complex. This interpretation of the thymidine *trans* effect is complicated by the likelihood that thymidine fluxes into the hepatocytes are mediated by more than one NT system (apparently by an Na^+-linked transporter and possibly by an equilibrative transporter). Thus, the *trans* effects here observed cannot be attributed unequivocally to the presence of an equilibrative transport system because it is not known whether the activity of the transport system that mediates the Na^+-linked thymidine flux might also be influenced by the intracellular concentration of thymidine. Thus, the acceleration of [^{3}H]thymidine influx by intracellular thymidine would be consistent with, but does not prove the participation of an equilibrative transport system in thymidine transport in these cells.

D. Dependency of NT on external sodium ions

In the present studies, the reduction in uridine and thymidine fluxes in hepatocytes that resulted from replacement of Na^+ by K^+ in the suspending medium suggests that a Na^+-linked, concentrative NT system(s) contributed to the fluxes. Intracellular uridine and thymidine concentrations achieved in the hepatocytes were about twice the extracellular concentration when cells were suspended in Na^+ medium (Figs. 8, 9, 13). In probing the ion dependence of cotransport systems, many investigators have replaced Na^+ salts in media with K^+, Li^+ and other salts, including choline chloride. While choline chloride has been the

classical NaCl replacement in simple salts media, this salt is not ideal because of problems with impurities, deliquescence and the limited range of choline salts available (Blackstock *et al.*, 1985). N-Methyl-D-glucammonium chloride has been used to replace NaCl in a number of studies (Reuss and Finn, 1975; Taylor and Rennie, 1987; Cook *et al.*, 1987). In this laboratory, NMG^+ replacement of Na^+ in cell suspension media was employed in demonstrating the activity of Na^+-linked, concentrative NT systems in leukemia L1210 cells (Dagnino *et al.*, 1987) and in IEC-6 intestinal epithelial cells (Jakobs and Paterson, 1986). N-Methyl-D-glucamine of high purity is available commercially.

A component of thymidine influx appeared to be Na^+-linked in that fluxes were reduced when Na^+ was replaced by K^+. However, membrane depolarization might have contributed to that reduction. The demonstration (Fig. 16) that the thymidine transport activity persisted in K^+ medium suggests that (i) K^+ may be a co-substrate, albeit a poor one, for the presumed cation-linked thymidine transporter, and (ii) a Na^+-independent NT system may also contribute to thymidine permeation. In K^+ medium, uptake of thymidine by hepatocytes during 5 min intervals was inhibited by NBMPR, indicating participation of a facilitated diffusion system in thymidine entry. The nature of the NBMPR-insensitive component of thymidine permeation in K^+ medium is unclear because a part of this activity might be attributable to the cation-dependent system, if K^+ were able to serve as a co-substrate in place of Na^+.

When NMG^+ replaced Na^+ in suspending medium, thymidine influx rates were increased and permeation was clearly concentrative (Fig. 15). While the reduction in thymidine uptake that resulted when K^+ replaced Na^+ in media argues for the presence of an Na^+-dependent NT system, the enhancement of thymidine uptake seen in NMG^+ medium (Fig. 16) does not sustain that argument, as it has done in other cell systems in this laboratory (Jakobs and Paterson, 1986; Dagnino *et al.*, 1987). Thus, it is possible that NMG^+ may serve in place of Na^+ as the co-substrate of a concentrative transport system that mediates the permeation of thymidine. This response was unexpected since NMG^+ did not replace Na^+ in the Na^+-linked, concentrative transport of formycin B in IEC-6 cells

(Jakobs and Paterson, 1986), nor in the Na^+-linked NT system of L1210 cells (Dagnino *et al.*, 1987). Neither of the latter systems transports thymidine and neither is sensitive to NBMPR. The effect of NBMPR on thymidine uptake by hepatocytes in NMG^+ medium (Fig. 15) was similar to that in Na^+ medium (Fig. 13). Thus, NMG^+ influenced the NBMPR-insensitive component of thymidine influx. The molar effect of NBMPR on thymidine uptake by hepatocytes was similar whether in Na^+ or NMG^+ medium. The concentration-effect relationship for NBMPR inhibition of thymidine transport in NMG^+ medium was not explored in the present study.

E. Pyrimidine nucleoside metabolism in hepatocytes

Of the quantities of uridine and thymidine that entered rat hepatocytes under the conditions of these experiments, less than 20% was converted to other products during a 10 min period. As shown in Table 6, phosphates accounted for about 8% of the cellular uridine-derived ^3H-activity after 10 min at 22 °C, and an unknown product represented a further 4%. Since no detectable radioactivity was associated with uracil on the chromatograms, it is reasonable to assume that the unknown product may be dihydrouracil or another metabolite beyond uracil in the catabolic pathway (Fig. 3). The apparent absence of uracil as a catabolite of [^3H]uridine in hepatocytes is consistent with the finding that the catabolism of uridine to uracil occurs predominantly in Kupffer cells (Holstege *et al.*, 1985).

The presence in some murine tissues, including liver, of free uridine pools at concentrations considerably above those of plasma was reported by Darnowski and Handschumacher (1986) and Darnowski *et al.* (1987) who also demonstrated the concentrative transport of uridine in splenocytes. Those results and the data on uridine metabolism reported here, support suggestions that the liver may act as a source of uridine for use by extrahepatic tissues (Hogans *et al.*, 1971; Tremblay *et al.*, 1977). The finding of the present study that thymine was the only apparent thymidine metabolite in hepatocytes, is consistent with the observation that levels of thymidine kinase activity in non-regenerating liver are low (Ferdinandus, *et al.*, 1971; Ungemach and Hegner, 1978).

F. Kinetics of uridine and thymidine transport

The data of Figs. 12 and 21 indicate that high-affinity and low-affinity systems for uridine and thymidine transport are expressed in rat hepatocytes. The kinetic constants for the two systems were obtained by a nonlinear regression analysis using a model-fitting computer program (Mcpherson, 1983) which resolved the kinetic constants for the two processes. Using the Michaelis-Menten equation, the proportions of high-affinity (low K_m) and low-affinity (high K_m) systems that contributed to uridine entry were derived to be 37% and 63% respectively. These values correlate with 35% for NBMPR-sensitive and 65% for NBMPR-insensitive component. Thus, it is reasonable to conclude that the high-affinity system is NBMPR-sensitive.

G. Conclusions and suggestions for further work

The present study has shown that rat hepatocytes possess binding sites with high affinity for NBMPR, an observation consistent with an earlier report (Plagemann and Wohlhueter, 1985) and that a component of uridine influx in rat hepatocytes is of high sensitivity to NBMPR. This study has also shown that a component of uridine influx in hepatocytes is Na^+-linked and is probably concentrative. A major part of the Na^+-linked uridine influx was of low sensitivity to NBMPR. Low K_m and high K_m processes contributed to uridine uptake in Na^+ medium. Uridine is poorly metabolized by hepatocytes in suspension.

The following major features of thymidine transport in rat hepatocytes were shown in this study:

(i) A cation-dependent, concentrative transport system contributed to thymidine entry. Remarkably, that system appears to accept NMG^+ as a co-substrate. The hepatocyte Na^+-linked NT system is distinct from the Na^+-linked, concentrative NT systems of IEC-6 cells and L1210 cells which do not transport thymidine and do not accept NMG^+ as an ionic co-substrate in place of Na^+. It is not clear whether the hepatocyte Na^+-linked NT system is partially inhibited by NBMPR.

(ii) A demonstration of counter-transport suggested the operation in hepatocytes of an equilibrative transport system that accepts thymidine as a substrate. That result would be consistent with the presence on hepatocytes of sites that bind NBMPR with high affinity. The NBMPR binding sites indicate that the hepatocytes express equilibrative, NBMPR-sensitive NT activity.

(iii) NT systems of high and low NBMPR sensitivity are involved in thymidine permeation in hepatocytes. As noted above, it is not yet clear if the Na^+-linked NT system is sensitive to NBMPR.

(iv) Thymidine is poorly metabolized by hepatocytes in suspension.

(v) Rate-concentration study of thymidine uptake by hepatocytes indicated that both low K_m and high K_m processes contributed to thymidine fluxes.

It is evident from this study that several permeation systems take part in the entry of the pyrimidine nucleosides into hepatocytes. An immediate extension of this study should be definition of the systems involved. The participation of Na^+-nucleoside co-transport systems seems likely. The kinetic parameters for the different transport systems should be derived. The contribution of non-mediated components of transport should be evaluated with such L-nucleosides as are available. Since the stimulation of thymidine uptake by NMG^+ was a major unexpected effect, it will be important to further investigate this phenomenon. A look at the driving mechanism for this process is necessary.

The use of dipyridamole and dilazep as potential inhibitors of NT in hepatocytes should be of interest. Study of the permeation of purine nucleosides and their analogues into hepatocytes will also be necessary in evaluating the specificity of the transport systems described in this study. Because the substrate specificities of concentrative uridine transport in murine splenocytes and enterocytes differ (Darnowski *et al.*, 1987), it will be necessary to see if the transport systems in hepatocytes behave similarly towards pyrimidine and purine nucleosides. The effects of the NT inhibitors on the intracellular disposition of both pyrimidine and purine nucleosides in these cells will be important in understanding the handling of these compounds by the liver.

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